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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : <b>C12N 1/19, 15/63, 15/66, 15/67, 15/81</b>		A1	(11) International Publication Number: <b>WO 99/10474</b> (43) International Publication Date: 4 March 1999 (04.03.99)
(21) International Application Number: PCT/US98/17592 (22) International Filing Date: 24 August 1998 (24.08.98)		(81) Designated States: CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority Data: 60/056,719 22 August 1997 (22.08.97) US		Published <i>With international search report.</i>	
(71) Applicant: SCRIPTGEN PHARMACEUTICALS, INC. [US/US]; 610 Lincoln Street, Waltham, MA 02451 (US).			
(72) Inventors: BRADLEY, John, D.; 25 Parkman Street #1, Brookline, MA 02146 (US). THOMPSON, Craig, M.; 20 Concord Avenue #17, Cambridge, MA 02138 (US). MOORE, Jeffrey, B.; 171 South Street #9B, Jamaica Plain, MA 02130 (US). WOBBE, Richard, C.; 57 Spring Street, Lexington, MA 02173 (US). HEALY, Judith, M.; 54 Shad Street, Lexington, MA 02173 (US). DONNELLY, Caroline, E.; 20 Cot Hill Road, Bedford, MA 01730 (US).			
(74) Agents: WILDMAN, David, E. et al.; Darby & Darby P.C., 805 Third Avenue, New York, NY 10022-7513 (US).			

(54) Title: REGULATED GENE EXPRESSION IN YEAST

(57) Abstract

The invention provides novel yeast cells comprising genes whose expression can be modulated by growth in the presence or absence of metal ions, methods for making such yeast cells, and methods of using such yeast cells for determining the requirement for expression of particular genes of the growth or viability of the yeast cells.

ATTORNEY DOCKET NUMBER: 10182-016-999  
SERIAL NUMBER: 10/032,585  
REFERENCE: BR

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## REGULATED GENE EXPRESSION IN YEAST

### Field of the Invention

5 The present invention relates to methods and compositions for regulated expression of specific genes in *Saccharomyces cerevisiae*. The invention can be used to identify and clone genes of interest and to identify antifungal agents using high-throughput screening techniques.

### 10 Background of Invention

The ability to regulate the expression of particular genes of interest is important for many purposes, including, for example, (i) investigation of the biological function of a particular gene product; (ii) design of variants of the gene product that are tailored for different ends; and (iii) identification of agents that influence the activity of the 15 gene product, including, e.g., inhibitors or activators. The ease of performing genetic and molecular manipulations in *S. cerevisiae* has made it an extremely useful experimental organism for regulated expression of recombinant genes. However, many gene expression systems based on *S. cerevisiae* are limited in their applicability by (i) the degree of regulation that can be achieved, i.e., the extent to which genes can be turned on and off, as well as the 20 timing of these events; (ii) the relative stability of certain gene products, which makes it difficult to quickly deplete the cell of a gene product; and (iii) potential metabolic side effects of the procedures used to trigger or initiate changes in gene expression.

Thus, there is a need in the art for *S. cerevisiae* expression systems in which gene expression can be tightly and efficiently regulated, with respect to both transcription of 25 the gene and accumulation of the protein product.

### Summary of the Invention

The present invention encompasses yeast strains in which expression of a particular protein (the "subject" protein) can be tightly regulated. The invention provides 30 *Saccharomyces cerevisiae* cells in which expression of the subject protein can be repressed

by exogenous metal. These cells comprise, for example:

- (i) a first gene encoding a transcriptional repressor protein, the expression of which has been placed under the control of a metal ion-responsive element, wherein expression of the repressor protein is stimulated by the addition of a metal ion to the 5 growth medium of the cells;
- (ii) a second gene encoding a subject protein, wherein expression of the subject protein is controlled by a promoter, the activity of which is inhibited by said repressor protein; and
- (iii) a third gene encoding a biomineralization protein, wherein the 10 third gene is inactivated and wherein inactivation of the third gene enhances the transcriptional response of the metal-responsive element to added metal ions.

In a preferred embodiment, the first gene is ROX1; the second gene is controlled by an ANB1 promoter; and the third gene is SLF1.

In another embodiment, the yeast cell comprises a fourth gene encoding a 15 protein that targets ubiquitin-containing polypeptides for degradation, where the fourth gene is placed under the control of a metal ion-responsive element. In a preferred embodiment, the fourth gene is the UBR1 gene.

The invention further comprises yeast cells in which expression of the subject protein is stimulated by exogenous metal ions. These cells comprise:

- 20 (i) a first gene encoding a subject protein, wherein expression of the gene encoding the subject protein is under the control of a metal ion-responsive element and is stimulated by the addition of a metal ion to the growth medium of the cells; and
- (ii) a second gene encoding a biomineralization protein, wherein the second gene is inactivated and wherein inactivation of the second gene enhances the 25 transcriptional response of the metal-responsive element to added metal ions.

In a preferred embodiment, the metal-responsive element is the Sc3451 promoter and the second gene is SLF1.

In another aspect, the invention relates to a method for the introduction of a subject gene under the control a predetermined promoter DNA sequence into a yeast cell 30 genome, comprising the steps of providing a shuffled gene fragment, where the fragment comprises a restriction enzyme cleavage sequence, ligating the shuffled gene fragment into

a vector, where the ligation results in the shuffled gene fragment being operably linked to a predetermined transcriptional control DNA sequence, cutting the vector with a restriction enzyme specific for the restriction enzyme cleavage sequence to yield a linearized vector, and transforming a yeast cell with the linearized vector.

5 The invention also provides methods for repressing or activating expression of a gene encoding a subject protein in *S. cerevisiae* to a predetermined level, comprising culturing the strains described above in the presence of metal, wherein the metal is present at sufficient concentration to activate the metal-responsive element so as to achieve the predetermined level of repression or activation of the gene.

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#### Brief Description of the Drawings

Figure 1 is a graphic illustration of the growth of yeast strains CTY145 and ZMY60 in increasing concentrations of copper sulfate. CTY145 is four-fold more tolerant to copper than ZMY60.

15 Figure 2 is a schematic illustration of the single- or double-round PCR strategy of the present invention that is used to construct a copper-inducible promoter element for any gene of interest. For single round PCR, primer pairs 1 and either 2a or 2b are used to produce the transforming DNA. For the double-round PCR, primer pairs 2a or 2b are used with additional primers corresponding to sequences located 400-1000 bp 20 upstream or downstream of the ATG start site to prepare long primers which are then used in a second round of PCR to produce transforming DNA.

Figure 3 is a schematic illustration of a PCR primer design strategy that can be used to render a gene encoding a protein of interest repressible by LexA. The upper panel shows the structure of the promoter complex that can be inserted upstream of the gene of 25 interest. The middle panel shows the location of the sequences corresponding to the PCR primers that can be utilized. The lower panel shows the resulting promoter cassette fused to the gene of interest which can be introduced into yeast.

Figure 4 is an illustration of the nucleotide sequence of the ZM195 plasmid (SEQ ID NO:20).

30 Figure 5 is an illustration of the nucleotide sequence of the ZM197 plasmid (SEQ ID NO:21).

Figure 6 is a schematic illustration of the PCR strategy used to generate shuffled genes for transformation into *S. cerevisiae*.

Figure 7 is a schematic illustration of the transformation mechanism by which shuffled genes are introduced into *S. cerevisiae*.

5 Figure 8 is a restriction map of the pCU19Srf vector (SEQ ID NO:22), showing the unique restriction enzyme cutting sites.

Figure 9 is a photograph of cell filtrates demonstrating the effect of deleting the SLF1 gene on biomineralization by yeast.

10 Figure 10 is a graph demonstrating the effect of copper sulfate on the growth of yeast strains which express and do not express the SLF1 gene.

Figure 11 is a graph showing growth curves for control and recombinant yeast strains which were grown in the presence of copper sulfate.

#### Detailed Description of the Invention

15 All patents, patent applications, publications and other materials cited herein are hereby incorporated by reference in their entirety. In the case of inconsistencies, the present description, including definitions, is intended to control.

As used herein, the term "transcriptional repressor protein" refers to a protein which either binds directly to a transcriptional control sequence or which binds in association 20 with other proteins or cofactors to a transcriptional control sequence, resulting in the repression of transcription of the protein encoding nucleotide sequence or sequences to which the transcriptional control sequence is operably linked.

As used herein, the term "transcriptional control sequence" refers to DNA sequences, such as initiator sequences, enhancer sequences, and promoter sequences, which 25 induce, repress, or otherwise control the transcription of protein encoding nucleic acid sequences to which they are operably linked.

As used herein, the term "metal-ion responsive element" refers to a transcriptional control sequence which is activated when in the presence of an appropriate concentration of metal ions.

30 As used herein, the term "inactivated", when referring to a gene, means that the gene cannot be transcribed, either due to deletion of the gene from a genome or by

disruption of its coding or regulatory sequences.

As used herein, the term "biomineralization protein" refers to a protein that promotes or catalyzes the conversion of ionic copper to a form insoluble in water, such as CuS.

5 As used herein, the term "shuffled gene fragment" refers to the nucleotide sequence around the ATG initiation codon of a gene, from about 400 nucleotides upstream of (i.e., 5' to) the ATG initiation codon of the gene to about 400 protein coding nucleotides downstream of (i.e., 3' to) the ATG initiation codon of the gene, wherein the orientation of the upstream and downstream sequences have been changed such that the ATG initiation 10 codon and the approximately 400 downstream protein coding nucleotides that follow the ATG codon in the wild-type gene are upstream to the approximately 400 noncoding nucleotides normally found adjacent and upstream of the ATG initiation codon. The shuffled gene fragment will typically contain a restriction enzyme cleavage sequence between the rearranged coding and noncoding nucleotide sequences.

15 As used herein, the term "restriction enzyme cleavage sequence" refers to a specific nucleotide sequence which is specifically recognized and cleaved by one or more restriction endonuclease enzymes.

As used herein, the term "operably linked" refers to the covalent attachment, typically of a transcriptional control sequence to a protein encoding nucleotide sequence, 20 such that transcription of the protein encoding nucleotide sequence is regulated or controlled by the transcriptional control sequence.

As used herein, the term "linearized vector" refers to the cleavage product of circular double stranded DNA molecule, or vector, which has been cleaved at a single site, yielding a linear double stranded DNA molecule.

25 The present invention encompasses methods and compositions for regulating the expression of a gene of interest in *Saccharomyces cerevisiae*. The invention provides recombinant yeast strains which comprise:

(i) a gene encoding a transcriptional repressor protein, the expression of which gene has been placed under the control of a metal ion-responsive element, so that 30 expression of the gene encoding the repressor protein is stimulated by the addition of a metal ion to the growth medium of the cells;

- (ii) a gene encoding a protein of interest, the expression of which gene is inhibited by the repressor protein described in (i); and
- (iii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the transcriptional 5 response to added metal ion.

In the above yeast cells (a large number of such clonal cells being collectively designated "repressing strains"), the gene of interest is expressed in the absence of added metal ion. When it is desired to decrease or eliminate expression of the gene of interest, metal ions are added to the medium, which stimulates expression of the repressor to a degree 10 that is dependent upon the concentration of added metal ions and represses transcription of the gene of interest.

The invention also encompasses yeast cells (a large number of such clonal cells being collectively designated "inducing strains") in which: (i) the gene of interest is operably linked to a metal ion-inducible transcriptional control sequence, so that expression 15 of the gene of interest is directly stimulated by addition of metal ions to the medium; and (ii) one or more genes involved in metal ion metabolism that have either been inactivated or overexpressed, depending on the gene, to enhance the transcriptional response to added metal ion.

The choice of yeast strain in which the above manipulations are performed is 20 important in practicing the invention. Suitable strains are those that tolerate the addition of metal ions to their culture medium at a sufficient concentration, and for a sufficient time period, to allow maximal expression of metal-inducible genes while maintaining cell viability and metabolism. Preferably, the growth rate of the strain should remain substantially unaffected for at least about 16 h after the addition of at least 750  $\mu$ M copper sulfate, most 25 preferably at least 1 mM copper sulfate. In addition, the strain should grow well and should be auxotrophic for common nutrients such as histidine, leucine, and uracil, to enable the use of, e.g., HIS3, LEU2, and URA3 as markers for genetic insertions. Suitable yeast strains include without limitation CTY145 and S288C (ATCC #26108).

In some embodiments, the repressing strains of the invention further comprise 30 a gene encoding a protein that targets ubiquitin-containing polypeptides for degradation via the ubiquitin degradation pathway, which, similar to the repressor gene, is expressed under

the control of a metal ion-responsive regulatory element. In these embodiments, the gene of interest is expressed as a fusion protein, which contains at its amino terminus additional amino acids comprising a sequence that targets the polypeptide for the ubiquitin degradation pathway. In this manner, addition of metal ions to the medium also stimulates degradation 5 of the protein of interest by the ubiquitin pathway, thereby depleting the protein from the cell. It will be understood, however, that some proteins of interest cannot be expressed in functional form as ubiquitin-targetable fusion proteins. Furthermore, overexpression of a ubiquitin-pathway gene may exert pleiotropic and potentially deleterious effects. Accordingly, the invention also encompasses repressing strains that do not overexpress a 10 ubiquitin pathway protein and in which the gene of interest is not expressed as a fusion protein.

In practicing the invention, any metal ion-responsive transcriptional control element may be used, including without limitation DNA sequences comprising the binding site for the ACE1 protein, which has been identified as the sequence spanning nucleotides 15 -105 to -148 of the CUP1 (metallothionein) promoter (Huitbregtse et al., *Proc.Natl.Acad.Sci.USA* **86**:65, 1989). Metal ion-responsive elements may be used singly or in tandem repeats, in direct or reverse orientation relative to a transcription start site, and may be combined with any compatible promoter such as, e.g., the HIS3 promoter. In conjunction with these elements, any suitable metal ion may be used to stimulate expression, 20 including without limitation Ag, Cu, Cd, Ni, Zn, and Fe ions. Suitable repressor proteins for use in the invention include without limitation ROX1, a heme-induced repressor of hypoxic genes (Genbank accession number #X60458) (Deckert et al., *Genetics* **139**:1149, 1995), LexA-CYC8 fusion proteins and LexA-TUP1 fusion proteins (Redd et al., *Cell* **78**:709, 1992). It will be understood that the choice of promoter sequences to be placed upstream of 25 the gene of interest will be determined by the particular repressor used. For example, when ROX1 is the repressor, the promoters directing expression of the gene of interest may be derived from, e.g., the ANB1, HEM13, ERG11, or OLE1 genes. The sequences of these genes are disclosed under the following Genbank accession numbers: #M23440 (ANB1); #S81592 (HEM13); #U10555, U00093 (ERG11); and #U42698, #J05676 (OLE1). When 30 the repressor contains bacterial LexA domains, the promoters directing the expression of the gene of interest may comprise sequences derived from the LexA operator. The sequence of

the LexA operator is 5'-TACTGATGTACATACAGTA-3' (Tzamarias et al., *Nature* **369**:758, 1994) (SEQ ID NO:1); a synthetic LexA operator may also be employed, comprising the sequence:

5' TCGAGTACTGTATGTACATACAGTACCATGACATACATGTATGTCATGAGCT-3'  
5 (U.S. Patent No. 4,833,080) (SEQ ID NO:2).

The genes involved in metal ion metabolism that may be inactivated to form the yeast strains of the present invention include without limitation SLF1, which is involved in the biomineralization pathway of copper (Genbank accession number U30375) (Yu et al., *Mol. Cell. Biol.* **16**:2464, 1996). In the case of SLF1, inactivation of the gene slows the 10 depletion of copper from the growth medium and thereby enhances the transcriptional response of the repressor-encoding gene to the added copper ions. The result is an increase in the time period in which a consistent copper regulation of gene expression can be maintained. Alternatively, genes encoding proteins such as, e.g., CTR1 (a metal ion transporter) can be overexpressed to increase the sensitivity of the transcriptional apparatus 15 to the added metal ion (Dancis et al., *J. Biol. Chem.* **269**:25660, 1994).

In the embodiments in which a ubiquitin-pathway protein is expressed under metal ion control, any ubiquitin-pathway protein may be expressed that will stimulate the degradation of an appropriately amino terminal tagged protein of interest. In one embodiment, the ubiquitin pathway protein that is linked to a metal ion-responsive element 20 is UBR1 and the amino terminal tag is a hybrid sequence comprising, in amino terminal-to-carboxyl terminal direction, ubiquitin and a 31-amino acid segment of the lac repressor protein (LacI), and may additionally include one or more epitope tags (Park et al., *Proc. Natl. Acad. Sci. USA* **89**:1249, 1992). In this embodiment, the hybrid protein (containing at its carboxyl terminus the protein of interest) is rapidly de-ubiquinated by yeast enzymes, 25 and the resulting hybrid protein (containing an arginine residue at its amino terminus) is re-ubiquinated by the UBR1 protein (in the presence of a metal ion) and targeted for degradation.

Moqtaderi et. al., *Nature* **383**:188, 1996, disclose a haploid yeast strain (ZMY60) carrying integrated copies of the ROX1 and UBR1 genes which were placed under 30 the control of the ACE1 promoter. Into this genetic background, a plasmid containing the ANB1 promoter driving expression of an in-frame fusion of ubiquitin, arginine, lacI,

hemagglutinin epitopes and the full length gene of interest was introduced. Addition of 500  $\mu$ M cupric sulfate ( $\text{CuSO}_4$ ) to the medium resulted in the repression of transcription of the gene of interest by ROX1 and rapid degradation of the ubiquitin-tagged protein. However, this strain is relatively genetically unstable resulting in frequent reversion to a copper-5 insensitive phenotype, is highly sensitive to the toxic effects of copper ions at concentrations above 250  $\mu$ M, and responds to copper ions for a relatively short time (in part, due to depletion of copper ions from the medium by biomineralization). The yeast strains of the present invention, by contrast, tolerate concentrations of copper ions of 1 mM or greater for extended periods of time. Furthermore, the yeast strains of the present invention exhibit 10 more stable phenotypes, due to the use of methods which employ double-crossover events for integration of engineered genes into the yeast genome (see, e.g., Examples 3 and 4 below).

In one set of embodiments, the invention provides a CTY145-based yeast strain in which: (i) the native ROX1 gene promoter has been replaced with a promoter 15 comprising a hybrid HIS3 promoter-ACE1 binding site; (ii) the native SLF1 gene has been deleted; and (iii) the gene of interest is controlled by an ANB1 promoter. Features (i)-(iii) are preferably achieved using a double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in addition, (iii) the native UBR1 gene promoter has been replaced with a promoter comprising a hybrid HIS3 promoter-20 ACE1 binding site; and (iv) a sequence which comprises an ANB1 promoter followed by a sequence encoding a hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

In another set of embodiments, the invention provides a CTY145-based yeast strain in which: (i) a gene has been introduced comprising a hybrid HIS3 promoter-ACE1 25 binding site placed upstream of sequences encoding a CYC8-LexA fusion protein; (ii) the native SLF1 gene has been deleted; and (iii) a gene of interest is controlled by a promoter comprising a LexA operator. Features (i)-(iii) are preferably achieved using a double-crossover strategy. In an alternate embodiment, the CTY145 strain has been modified as in (i) and (ii) above, and, in addition, (iii) the native UBR1 gene promoter has been replaced 30 with a promoter comprising a hybrid HIS3 promoter-ACE1 binding site; and (iv) a sequence which comprises a LexA operator-containing promoter followed by a sequence encoding a

hybrid polypeptide comprising ubiquitin, a LacI fragment, and an epitope tag is fused to the 5' end of the protein-coding sequence of a gene of interest.

In another set of embodiments, the invention provides a CTY145-based yeast strain in which (i) the gene of interest is controlled by the Sc3451 promoter and (ii) the native 5 SLF1 gene has been deleted. The Sc3451 promoter was constructed by cloning an ACE1 binding site (5'-TAAGTCTTTTGCTGGAACGGTTGAGCGGAAAAGACGCATC-3') (SEQ ID NO:3) upstream of the TATAA sequence at an EcoRI site in plasmid YIp55-Sc3370 (Struhl et al., *Mol. Cell Biol.* 7:104, 1987).

## 10 METHODS

In practicing the present invention, many conventional techniques in molecular biology, microbiology, and recombinant DNA, are used. Such techniques are well known and are explained fully in, for example, *Current Protocols in Molecular Biology*, Volumes I, II, and III, 1997 (F.M.. Ausubel ed.); Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; *DNA Cloning: A Practical Approach*, Volumes I and II, 1985 (D.N. Glover ed.); *Oligonucleotide Synthesis*, 1984, (M.L. Gait ed.); *Nucleic Acid Hybridization*, 1985, (Hames and Higgins); *Transcription and Translation*, 1984 (Hames and Higgins eds.); *Animal Cell Culture*, 1986 (R.I. Freshney ed.); *Immobilized Cells and Enzymes*, 1986 (IRL Press); Perbal, 1984, *A Practical Guide to Molecular Cloning*; the series, *Methods in Enzymology* (Academic Press, Inc.); and *Methods in Enzymology* Vol. 154 and Vol. 155 (Wu and Grossman, and Wu, eds., respectively). Insertion of nucleic acids (typically DNAs) comprising the sequences of the present invention into a vector is easily accomplished when the termini of both the DNAs and the vector comprise compatible restriction sites. If this cannot be done, it may be necessary to modify the termini of the DNAs and/or vector by digesting back single-stranded DNA overhangs generated by restriction endonuclease cleavage to produce blunt ends, or to achieve the same result by filling in the single-stranded termini with an appropriate DNA polymerase.

Alternatively, any site desired may be produced, e.g., by ligating nucleotide 30 sequences (linkers) onto the termini. Such linkers may comprise specific oligonucleotide sequences that define desired restriction sites. Restriction sites can also be generated by the

use of the polymerase chain reaction (PCR). See, e.g., Saiki *et al.*, 1988, *Science* 239:48. The cleaved vector and the DNA fragments may also be modified if required by homopolymeric tailing.

The nucleic acids may be isolated directly from cells. Alternatively, the 5 polymerase chain reaction (PCR) method can be used to produce the nucleic acids of the invention, using either chemically synthesized strands or genomic material as templates. Primers used for PCR can be synthesized using the sequence information provided herein and can further be designed to introduce appropriate new restriction sites, if desirable, to facilitate incorporation into a given vector for recombinant expression.

10 Methods for yeast transformation, integration of genes into the yeast genome, and growth and selection of yeast strains are fully described in, e.g., *Current Protocols in Molecular Biology*, Vols. 1 and 2, Ausubel *et al.*, eds., John Wiley & Sons, New York (1997). The use of URA3 for the production of multiply disrupted yeast strains is disclosed in Alani *et al.*, *Genetics* 116:541, 1987.

15 A preferred method for the transformation of *S. cerevisiae* is as follows. Yeast strains are cultured overnight in YPD (yeast extract, peptone, dextrose) medium at about 30°C. The resulting culture is diluted to an  $A_{600}$  of about 0.2 in about 200 ml YPD medium and incubated at about 30°C until the  $A_{600}$  reaches approximately 0.8. The cells are pelleted by centrifugation and are washed in about 20 ml sterile water. The pelleted yeast cells are 20 then resuspended in about 10 ml TEL (10mMTris pH7.5, 1 mM EDTA, 0.1 M LiAcetate pH 7.5) buffer. The cells are pelleted by centrifugation and again resuspended in about 2 ml TEL. About 100 µg of well-sheared single stranded DNA and plasmid DNA are added to an eppendorf tube. To this tube is added about 100 µl of competent yeast cells, followed by mixing. To the cell/DNA mixture is added about 0.8 ml of 40% PEG-3350 in TEL, followed 25 by thorough mixing. This mixture is incubated for about 30 minutes at 30°C, followed by a heat shock for 20 minutes at 42°C. The mixture is centrifuged to remove the supernatant and pellet the cells. The yeast cell pellet is washed with about 1 ml TE, pelleted again by centrifugation, and then plated on selective media.

30 A preferred method for the extraction of genomic DNA from *S. cerevisiae* for PCR is as follows. A 5 ml overnight yeast strain culture grown in YPD at 30°C is spun out by centrifugation and washed once in 1 ml Tris pH 7.5/1 mM EDTA (TE) buffer. The cells

are pelleted again by centrifugation and resuspended in 0.2 ml Extraction Buffer (2% Triton X100, 1% SDS, 100 mM NaCl, 10mM Tris pH 7.5 and 1mM EDTA) plus 0.2 ml phenol/chloroform/isoamyl alcohol. About 0.3 g of acid washed glass beads are added. This mixture is vortexed (i.e., agitated vigorously) for 30 minutes. 0.2 ml TE buffer is then added.

5 The mixture is centrifuged and the aqueous phase is removed. The DNA is precipitated from the aqueous phase with two volumes of ethanol. The precipitate is pelleted by microcentrifugation, and resuspended in 50  $\mu$ l TE plus 5  $\mu$ g/ml RnaseA enzyme. The resulting preparation can be diluted to a desired concentration, or used directly for PCR reactions.

10 For assaying the effects of copper ions on recombinant yeast strains, wild type and recombinant strains are grown in 5 ml of CSM media on a roller drum incubation apparatus at 30°C for 18 to 20 hours. Cultures are diluted to an  $A_{600}$  (absorbance of light at 600 nm) of about 0.02 in 5 ml CSM media without or with various concentrations of CuSO<sub>4</sub> (10  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 250  $\mu$ M, 1mM and 2 mM) for 18 to 20 hours. The  $A_{600}$  of the 15 various samples is read and recorded.

Yeast strains are tested by a time course in the presence and absence of copper to determine if the depletion of target gene product is fungistatic (i.e., inhibitory to growth) or fungicidal (i.e., yeast killing). Cultures are started from a single yeast colony in CSM media (5 ml) and grown at 30°C for 18 -20 hours in a roller drum. Cultures are diluted in fresh 20 media to a final volume of 10 ml at an  $A_{600}$  of about 0.25 and allowed to grow at 30°C for 1 hour. The cultures are split into two aliquots and 1 mM CuSO<sub>4</sub> is added to one of the aliquots. A sample of 300  $\mu$ l is immediately taken from each culture aliquot as the zero time point. Other similar samples are taken at 1, 3, 5, 7 and 24 hours after CuSO<sub>4</sub> addition. Alternatively, the cultures can be diluted to an  $A_{600}$  of about 0.1 and allowed to grow for 3 25 hours, at which time the cultures are diluted again to an  $A_{600}$  of about 0.02, after which 1 mM CuSO<sub>4</sub> is added. To measure the absorbance of a yeast culture, typically two-hundred microliters of each sample is taken and added to a 96-well flat bottom polystyrene plate, which is then inserted into a plate reader where the absorbance at 595 nm is measured. A growth curve can be generated from these readings.

30 When plating cells on YPD medium for analysis of CFU number, typically 100  $\mu$ l of each sample is serially diluted in 900  $\mu$ l sterile water.

Plating dilutions for yeast cultured without copper ions and for wild type yeast cultured in the presence of copper ions are from  $10^{-3}$  to  $10^{-6}$ . Plating dilutions for time points 0, 1, 3, 5, and 7 hrs for recombinant yeast cultured in the presence of copper ions range from  $10^{-2}$  to  $10^{-5}$ . Plating dilutions for any 24 hour time points for yeast cultured in the presence of copper ions ranges from undiluted to  $10^{-2}$ .

Typically, about one-hundred microliters of each dilution is plated on YPD agar plates and incubated at 30°C for 48 hrs. Colonies are counted and recorded. Calculations are made to convert colony counts to CFU/ml of original culture medium.

## 10 APPLICATIONS

The yeast strains of the present invention find use for:

(i) *Rapid and efficient determination of whether a particular gene of interest can serve as a potential target for discovery of antifungal drugs.* This is achieved by assessing whether the rapid depletion from yeast cells of a particular gene product (using 15 the "repressing strains" described above) leads to slowing of cell growth or cell death. Since the most effective drugs are those whose effect is rapidly fungicidal, a gene product whose depletion leads to cell death would be a potential target for antifungal drugs. Because the degree of the reduction in the amount of the gene product can be controlled by the concentration of metal added, it is further possible to determine the degree of reduction of 20 the gene product necessary to cause cell death.

(ii) *Identification of target gene products whose rapid depletion leads to increased sensitivity to known antifungal drugs.* This phenomenon could also identify potential synergies between known drugs and newly discovered antifungal compounds (see below).

25 (iii) *Rapid cloning of functionally complementary genes from other organisms, including pathogenic fungi such as *C. albicans* and *A. fumigatus*.*

(iii) *Development of libraries of strains, each of which contains a different gene which is either positively or negatively regulated by copper.* Such libraries are useful for identifying targets for antifungal drugs whose mechanism of action is unknown. For 30 example, if stimulation or repression of expression of a particular gene leads to decreased and increased sensitivity, respectively, to a particular drug, then the gene is likely to be involved

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in mediating the *in vivo* action of the drug.

(iv) *Development of high-throughput screening methodologies to detect antifungal compounds.* Such compounds may be found in, for example, natural product libraries, fermentation libraries (encompassing plants and microorganisms), combinatorial libraries, compound files, and synthetic compound libraries. For example, synthetic compound libraries are commercially available from Maybridge Chemical Co. (Trevillet, Cornwall, UK), Comgenex (Princeton, NJ), Brandon Associates (Merrimack, NH), and Microsource (New Milford, CT). A rare chemical library is available from Aldrich Chemical Company, Inc. (Milwaukee, WI). Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available from, for example, Pan Laboratories (Bothell, WA) or MycoSearch (NC), or are readily producible. Additionally, natural and synthetically produced libraries and compounds are readily modified through conventional chemical, physical, and biochemical means (Blondelle et al., *TibTech* **14**:60, 1996).

15 The following examples are intended as non-limiting illustrations of the present invention.

**Example 1: Construction of a Copper-Tolerant Yeast Strain Containing a Copper-Regulated ROX1 Gene**

20 The following experiments were performed to: (i) examine the copper sensitivity of different yeast strains; and, (ii) to create a copper-dependent yeast strain containing a copper-regulatable ROX1 gene.

The copper sensitivity of yeast strain CTY145 was compared with that of ZMY60 by growing each strain in the presence of increasing amounts of added copper and 25 measuring cell number at increasing times. After 16 hours of growth, the highest concentration of copper at which the log-phase growth rate of ZMY60 was maintained unaffected was 250uM. (Figure 1). By contrast, the highest concentration of copper at which the growth rate of CTY145 was maintained was 1 mM. Thus, CTY145 is at least four-fold more tolerant to copper than ZMY60.

30 A strain based on CTY145 that contains a copper inducible ROX1 was constructed as follows. Using a conventional lithium acetate/polyethylene glycol technique,

CTY145 was transformed with approximately 0.1 ug of plasmid pZM195 (Figure 4), which was linearized with the restriction enzyme AflII. The plasmid contains a metal responsive element comprising HIS3 promoter sequences fused to ACE1.

Integration of the plasmid into the genome was monitored by the ability of the 5 cells to grow on -URA plates (growth of the yeast strains in this medium requires the functional URA3 gene contained on the plasmid). After 48 hours at 30°C, the transformants were inoculated onto fresh -URA plates and regrown for an additional 48 hours at 30°C. Well spaced individual colonies were picked and inoculated to 5 ml YPD media, grown overnight at 30°C, and 5 µl of the culture was inoculated into 5-FOA plates. In the presence 10 of a functional URA3 gene, 5-FOA is converted to a toxin which kills cells. Therefore, the only cells that survive are those that have lost the URA3 gene by recombination.

Integration of ZM195 into the genome could occur in any of the following ways: (1) The original integration could occur non-specifically. In this case, the 5-FOA-induced deletion of URA3 would have no effect. (2) The original incorporation could occur 15 specifically, and the subsequent 5-FOA induced deletion would result in a return to the original promoter sequence. (3) The original integration could occur specifically, and the subsequent 5-FOA induced deletion of URA3 could lead to the correct insertion of the copper-inducible promoter directly upstream of the ROX1 open-reading frame (ORF).

To detect the correct promoter insertion, three PCR primers were designed: 20 ROX-A (5'-TCACACAAAAGAACGCAG-3')(SEQ ID NO:4), corresponding to a sequence from the region of the original promoter immediately 5' to the first ATG of the ORF; ROX-B (5'-GATGACAGCTGTGGTAGG-3')(SEQ ID NO:5), the reverse complement of a sequence in the ORF of ROX1 which is not present in ZM195; and ROX-C (5'-TCTTGCCATATGGATCTG-3')(SEQ ID NO:6), a sequence internal to the copper inducible 25 promoter. For possibilities 1 and 2 above, PCR amplification of genomic DNA with ROX-A and ROX-B would lead to a 601 base pair (bp) product, and PCR amplification with ROX-B and ROX-C would yield no product. For possibility 3, the correct insertion, PCR using ROX-A and ROX-B would yield a 2628 bp product, and PCR with ROX-B and ROX-C 30 would yield a 785 bp product. PCR analysis identified a strain that had undergone the correct rearrangements. This strain was designated CUY101.

To bring the UBR1 gene under the control of the copper inducible promoter,

HIS3-ACE1, the above-described procedure was repeated using CUY101 as the starting strain. The ZM197 plasmid (Figure 5) that had been linearized by digestion with the restriction enzyme AatII was introduced into the cells. To identify cells in which the correct promoter insertion had occurred, three PCR test primers were designed: UBR-A (5'-  
5 ATCTTCGGACAAAGGCAG-3') (SEQ ID NO: 7); UBR-B (5'-  
GTGTAATTCGGGATCG-3') (SEQ ID NO:8) and ROX-C (5'-  
TCTTGCATATGGATCTG-3') (SEQ ID NO:9). PCR analysis is used to identify one culture which has undergone the correct rearrangements. This strain is designated CUY103.

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**Example 2: Construction of a Yeast Strain Containing A Deletion of SLF1**

In practicing the present invention, it is preferred that copper regulation of expression be maintained over a relatively long time period, i.e., for several days. The transient effect of copper in wildtype yeast is due at least in part to the fact that yeast cells  
15 are able to biominerlize copper. Thus, over time wild-type yeast cells deplete the medium of copper and the effect on expression is lost. If biominerlization activity is ablated, then the extracellular copper levels should remain nearly constant over time.

The only known gene in the yeast copper ion biominerlization pathway is the SLF1 gene. Inactivation of the SLF1 gene has been shown to result in cells which are  
20 slightly more sensitive to copper but are unable to efficiently deplete copper from the media (Yu et al., *Mol. Cell Biol.* **16**:2464, 1996). Therefore, the SLF1 gene in the yeast strains described in Example 1 above was inactivated.

A construct was created for a two-step knockout of the SLF1 open reading frame. Primers SLF-E (5'-GCGCTGCAGGTGACTTAGCAGGCAGTTGAAC-3') (SEQ  
25 ID NO:10) and SLF-F (5'-GCGCTGCAGGCATGCACTCCTTCCAATTGTGC-3') (SEQ ID NO:11) were used to amplify the 3'- untranslated region of SLF1 using genomic DNA as template. The SalI/SphI fragment of the PCR product was subcloned into SalI/SphI-digested pUC19 plasmid (Genbank accession no. M77789). This recombinant plasmid was designated pSLF3'. Similarly, primers SLF-G (5'-  
30 GCGAGCTCGGTACCCATAACCCCTAACTCTAG-3') (SEQ ID NO: 12) and SLF-H (5'-GC GGATCCCGGGCTCTCGTTATTAACG-3') (SEQ ID NO:13) were used to

amplify the 5'- untranslated region of SLF1, and the SacI/BamHI or KpnI/BamHI fragment was cloned into SacI/BamHI or KpnI/BamHI digested pSLF3' to produce pSLF3'5'. The 5.5 kb BamHI/XbaI insert of pDJ20, which contains the yeast URA3 gene and bacterial kanamycin resistance gene flanked by a direct repeat of the *Salmonella* HisG sequence, was 5 subcloned into XbaI/BamHI digested pSLF3'5' to create pSLFKO. Plasmid pDJ20 is derived from the plasmid pSP72 (Promega, Madison, WI) into the BamHI site of which has been cloned the approximately 5.5. kb insert consisting of the following elements:

| hisG | URA3 | kanamycin resistance | hisG |

The hisG elements are present as a tandem repeat. Plasmids containing this element can be 10 transformed into bacteria for amplification; selection with kanamycin helps to avoid unwanted recombination between the two hisG regions in bacteria which would result in the loss of the *S. cerevisiae* URA3 gene. The hisG, URA3 and kanamycin genes are well-known in the art and can be assembled in this order by conventional techniques in molecular biology, and do not need to be obtained from plasmid pDJ20.

15 This plasmid (pSLFKO) was digested with SphI and EcoRI and transformed into strains ZMY60, CTY145, CUY101, and CUY103 using a conventional lithium acetate/polyethylene glycol technique, as described above in the Methods section. Integration of the plasmid into the genome of each yeast strain was monitored by the ability of the strain to grow on (-)URA plates. After 48 hours at 30°C, the transformants were 20 inoculated onto fresh (-)URA plates and regrown for an additional 48 hours at 30°C. Well spaced individual colonies were inoculated into 5 ml YPD media, grown overnight at 30 °C, and 5 µl of the culture was inoculated onto 5-FOA plates.

In the presence of a functional URA3 gene, 5-FOA is converted to a toxin 25 which kills cells. Therefore, the only cells that survive are those which have lost the URA3 gene by recombination. Either of the following could occur: (1) Non-specific integration of the linear DNA containing the 5'-HisG-URA3-kanR-HisG-3'NTS fragment could occur, followed by deletion of the region between the HisG repeats; this would result in a 5'NTS-HisG-3'NTS integration at some random spot. (2) Alternatively, specific integration of the linear DNA containing the 5'NTS-HisG-URA3-kanR-HisG-3'NTS sequence could 30 occur, followed by deletion, which would result in a deletion/insertion in which the entire ORF of SLF1 has been deleted and a single copy of the HisG element has been left in its

place.

To confirm that the correct genetic alteration occurred, PCR was performed using the following sets of primers: (i) HISGCH (5'-GATTGGTCTCTACCGGC-3') (SEQ ID NO: 14) and SLF-D (5'-GACAGTATCGTAATTACG-3') (SEQ ID NO: 15); and (ii) a 5 primer comprising the reverse complement of primer HISGCH and SLF-D as above. Alternatively, PCR with SLF-A (5'-CTAACTCTAGCTGCATTG-3') (SEQ ID NO:24) (or SLF-G) and SLF-D (or SLF-F) could be used to produce a diagnostic shift in product length after PCR. The SLF1 deleted version of CTY145 is designated CUY104; the SLF1 deleted version of CUY101 is designated CUY105; the SLF1 deleted version of CUY103 is 10 designated CUY106; and, the SLF1 deleted version of ZMY60 is designated CUY107.

Cultures of yeast strains CTY145, CUY101, CUY103, CUY104, CUY105, and

15 CUY106 were cultured in 5 ml of complete synthetic media (CSM) supplemented with 500 mM CuSO<sub>4</sub> at 30 °C in a rollerdrum apparatus at a speed of approximately 60 revolutions per minute. At 24 and 48 hours, the cultures were pelleted and resuspended in fresh CSM media containing 500 mM CuSO<sub>4</sub>. After 96 hours of incubation, cells were collected onto filter paper and the supernatant was removed by suction through the filter paper. Biomineralization is inferred by the presence of a darkened cell pellet, indicating the 20 biomineralization of the soluble copper to copper sulfide (CuS) which has been shown to be deposited on the cell surface. Strains CTY145, CUY101, and CUY103 (A, B, and C, respectively in Figure 9) contain the wild-type SLF1 gene, as demonstrated by their dark color. Strains CUY104, CUY105, and CUY106, in which the SLF1 gene has been deleted, show considerably lighter coloration after collection on filter paper, indicating an ablation 25 of copper biomineralization activity.

25 Single colonies of each yeast strain described above were picked from YPD plates and grown overnight in YPD media at 30 °C with shaking. Cultures were diluted to an absorbance at 600 nm of 0.02 in CSM media containing various concentrations of CuSO<sub>4</sub>. Cultures were grown for 24 hours at 30 °C in a rollerdrum apparatus at approximately 60 revolutions per minute. The absorbance of each culture at 600 nm, which is a measure of cell 30 density (i.e., the number of cells in a culture) was measured. The results of the cell density assays are shown in Figure 10, and is expressed as a percentage of the cell density achieved

in cells with no added copper sulfate.

**Example 3: Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter**

5

The following procedures were performed to stably replace the promoter element of any yeast gene of interest. The strategy is designed to avoid: (1) the use of URA3 as a selectable marker, which precludes its use in future selection procedures; (2) the requirement for a naturally occurring unique restriction site in the coding sequence of the 10 subject gene; (3) the need for multiple subclonings; and (4) the need for constant maintenance of URA3 selection in order to prevent loss of the inserted sequence, which would result in restoration of the original promoter elements.

A single or double-PCR strategy was devised. Instead of a single crossover event, the method requires that a double crossover occur in order to achieve integration into 15 the yeast genome. Although a double crossover event is less likely to occur, once it has occurred the resulting transformed haploid yeast strain does not have to be maintained under selection. HIS3 is used as a marker to avoid using URA3 unnecessarily; in addition, the HIS3 gene is relatively short and therefore comparatively easy to amplify.

A plasmid designated pCU3 was constructed which contains a functional 20 HIS3 gene (including upstream sequences) in inverted orientation to, and upstream from, the ANB1 promoter. The ANB1 promoter was fused upstream (i.e., 5' to) sequences encoding ubiquitin tag elements. For this purpose, the BamHI/PstI fragment of pUC8-Sc2676 was subcloned into pUC19. Then, the EcoRI-KpnI fragment of ZM168, which contains the ANB1 promoter and the ubiquitin tag regions, was subcloned into the plasmid.

25 Primers were designed for use in either a one-step or two-step PCR strategy. (Figure 2).

*One-Step PCR Strategy:* 100-mer oligonucleotides were synthesized. Primer 1 contains 80 bp of target sequence from the gene of interest, which is obtained from knowledge of the DNA sequence immediately 5' to the protein-coding sequence of the gene, 30 together with 20 bp of plasmid sequence. A second set of primers comprising sequences from the non-coding strand is synthesized. These oligonucleotides, which are designated

either Primer 2b (5'-CCAGACTACGCTTCGATATCG-3') (SEQ ID NO: 16) ("+Tag") or Primer 2a (5'-CACACTAAAACATCGATATT-3') (SEQ ID NO: 17) ("NO TAG"), are then fused to 18-20 bp of the protein-coding sequence of a gene of interest, beginning with the initiator ATG codon. In this case, "Tag" refers to the presence or absence of the ubiquitin tag.

Primer pairs 1 and 2a or 1 and 2b are used to amplify a DNA fragment from pCU3, producing a fragment consisting of genomic 5'NTS (non-translated sequence) followed by HIS3 in an inverted orientation, ANB1 promoter, and either a fragment of the ORF or a tag sequence fused in frame to a fragment of the ORF. Transformation of haploid yeast strains with these sequences, followed by double crossover, leads to integration into the genome. This results in insertion of HIS3, the ANB1 promoter and (in some cases) the tag sequence 5' to the gene of interest.

Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby considerably lessening the likelihood that the inserted sequence will be spontaneously deleted. After selection with HIS3, the presence and orientation of the insert is confirmed using PCR. Because the integration requires a double crossover, selection by HIS selection should not be required to maintain the genotype.

*Two-Step PCR Strategy:* For the two step strategy, Primer 2a or 2b is fused to 18-20 bp of the ORF of a gene of interest, beginning with the initial ATG of the ORF. Either primer, and a second primer comprising 18-20 nucleotides that is the reverse complement of a sequence 400-1000 bp downstream, are used to amplify a 400-1000 bp fragment that is the reverse complement of the ORF and has a 3' tag complementary to the sequence in pCU3 such that the sequence is fused in frame to the tags or is fused in frame in place of the tags.

The fragment corresponding to the opposite end is produced by fusing a primer designated "Universal HIS3-2STEP" (5'-CAGGCATGCAAGCTTGGCGT-3') (SEQ ID NO:18) to an 18- to 20-mer representing the reverse complement immediately 5' of the starting ATG of the ORF. This fragment is used in conjunction with a primer identical to 18-20 nucleotides comprising a sequence 400-1000 bp 5' to the starting ATG to amplify a fragment whose 3' end is complementary to the 3' end of the HIS3 gene in pCU3.

The two fragments are then used to amplify pCU3, producing a fragment

comprising a length of genomic 5'NTS followed by HIS3 in inverted orientation, ANB1 promoter and either a length of the ORF or a tag sequence fused in frame to a length of the ORF. Transformation with this sequence and double crossover leads to integration into the genome, which results in the insertion of HIS3, the ANB1 promoter and (in some 5 cases) the tag sequence. Using this approach, no DNA sequence is lost and no sequence is duplicated, thereby greatly lessening the likelihood of spontaneous deletion. After selection with HIS3, the presence and orientation of the insert is confirmed with PCR. Because the integration requires a double crossover, HIS selection should not be required to maintain the genotype.

10

**Example 4: Alternative Method for Stable Replacement of the Promoter Element of Any Gene of Interest with a Copper-Inducible Promoter**

PCR primers are designed to amplify sequences of the target gene to result in 15 a "shuffled gene" arrangement (Figure 6) in the vector pCU19Srf (Figure 8). The PCR primers that can be used will vary from 18 to 36 nucleotides in length, and will preferentially have a GC content of at least 50 percent. When primers, which are 18 to 36 nucleotides in length have a low GC content, the primers can be made 3 to 6 nucleotides longer than those with at least a 50% GC content.

20 PCR primers are dissolved in sterile water at a concentration of 1 mg/ml. Genomic DNA from *S. cerevisiae* is diluted to various concentrations in sterile water. Taq DNA polymerase (Promega Biotech, Madison, WI) is typically used for the primary PCR reaction, but other thermostable polymerases, such as Vent polymerase (New England Biolabs, Beverly, MA) or Pfu polymerase (Stratagene, Carlsbad, CA) can also be used.

25 The shuffled gene of interest is generated by performing two primary PCR reactions. In one, a portion of the target gene which starts about 400 base pairs upstream of the ATG start codon and ends just upstream of the ATG start codon is amplified. In the second primary PCR reaction, a portion of the target gene that starts at or that is just upstream of the ATG start codon and that ends about 400 base pairs downstream of the ATG start 30 codon is amplified. A typical primary PCR reaction will include 10 µl of 10X Taq buffer, 10 µl of 25 mM deoxynucleoside triphosphates, 10 µl of 25 mM MgCl<sub>2</sub>, 1 µl of *S. cerevisiae*

genomic DNA, and 1  $\mu$ l of primer pairs (Primers 1 and 2, or primers 3 and 4) at 100  $\mu$ g/ml, and 66  $\mu$ l of sterile water.

Typically, primer 1 will consist (from the 5' to the 3' end) of a seven nucleotide sequence, then a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the top strand of the gene of interest about 400 base pairs upstream of the ATG start codon of the target gene. The seven nucleotides at the 5' end of primer 1 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 4.

Primer 2 will have the sequence of the bottom strand of the gene of interest, 10 just upstream of the ATG start codon, and will comprise about 18 to about 21 nucleotides.

Primer 3 will have the sequence of the top strand of the target gene either at or very close to the ATG start codon.

Primer 4 will consist of a seven nucleotide sequence at the 5' end, followed by a NotI restriction endonuclease cleavage site (or a restriction site susceptible to cleavage 15 by another rare-cutting enzyme), followed by 10 or 11 nucleotides which are identical to the bottom strand of the gene of interest about 400 base pairs downstream of the ATG start codon. The seven nucleotides at the 5' end of primer 4 are complementary to the 7 nucleotides immediately 3' of the NotI site in primer 1.

Typically, the reaction is initiated by heating the reaction mixtures to 94  $^{\circ}$ C 20 for 3 to 5 minutes, followed by the addition of 5 units of Taq DNA polymerase. The reaction is then thermocycled reaction mixture through 1 minute at 94  $^{\circ}$ C, 1 minute at 50  $^{\circ}$ C, and 2 minutes at 72  $^{\circ}$ C for 30 cycles, after which the temperature is reduced to 4  $^{\circ}$ C. The PCR products are run on an agarose gel to determine the conditions that produced a fragment of the appropriate size, typically about 400 base pairs. Modifications of various parameters of 25 the method in order to optimize reaction conditions, such as altering annealing temperatures, salt concentrations, and the like are within the skill of the ordinarily skilled worker.

The secondary PCR reaction uses the primary PCR reaction products as DNA templates. The one end of each of the two primary PCR products are homologous, and when melted, will anneal to each other over a stretch of about 21 base pairs. The primers used for 30 the secondary PCR reaction are primers 2 and 3 used in the primary PCR reactions. Use of these primers will anneal to the ends of the annealed template DNA and allow the PCR

reaction to produce a shuffled gene reaction product.

A typical secondary PCR reaction will include 10  $\mu$ l of 10X Pfu buffer, 10  $\mu$ l of 25 mM deoxynucleoside triphosphates, 1  $\mu$ l of primer pairs (i.e., primers 2 and 3 from the primary PCR reaction) at a concentration of 100  $\mu$ g/ml, 77  $\mu$ l of sterile water, various 5 dilutions of the primary PCR products, and 1  $\mu$ l of Pfu polymerase, comprising 2.5 activity units. The PCR conditions are identical to those to be used in the primary reactions, except that the initial heating of the tubes to 94  $^{\circ}$ C for 3 to 5 minutes is omitted.

The secondary PCR product is electrophoresed through an agarose gel, according to methods well-known in the art, and the appropriate band (of about 800 to 900 10 base pairs) is cut out. The DNA is then extracted from the gel using, e.g., the Gene Clean kit (Bio101, Vista, CA). The extracted, purified DNA is then used for ligation into the vector pCu19Srf (Figures 8).

To perform the ligation, the PCR-Script kit from Stratagene (La Jolla, CA) is used. pCu19Srf is cut with SrfI restriction endonuclease. The ligation mix contains 100 15 ng of SrfI cut vector DNA, 1  $\mu$ l of 10X PCR Script buffer, 0.5  $\mu$ l ATP, 4 to 6  $\mu$ l of insert DNA, containing from 100 to 500 ng, 1  $\mu$ l of T4 DNA ligase, and 1  $\mu$ l of SrfI. All reagents are provided in the PCR Script kit except for the DNA. However, such reagents are well-known and commercially available from other sources. Three  $\mu$ ls of the ligation reaction is transformed into competent DH5 $\alpha$  cells, which can be obtained from Gibco/BRL (Rockville, 20 MD), and the cells are plated on LB medium with 100  $\mu$ g/ml ampicillin. The plates are incubated at 37  $^{\circ}$ C for 16 to 18 hours. Single colonies are chosen for restriction enzyme digestion and analysis of the resulting fragments to identify a clone which contains the insert in the proper orientation. A colony that is identified as containing a plasmid with the insert in the proper orientation is selected and is amplified by culturing. The insert-containing 25 plasmid is purified from the bacterial host using the DNA isolation procedure of the Qiagen DNA preparation kit (Qiagen, Hilden, Germany) or other well-known methods for plasmid purification. The purified DNA is digested with NotI restriction endonuclease (or another rare-cutting endonuclease whose restriction site has been engineered into the shuffled gene). The endonuclease is inactivated by heating for 20 minutes at 65  $^{\circ}$ C. The purified, cut DNA 30 is then used to transform *S. cerevisiae*.

Strain CUY106 (Ace-ROX1, AceUBR1, deltaSLF1, his3delta200, leu2-3,112,

ura3-52) is transformed by standard methods with the NotI digested plasmid DNA (Figure 7). Cells are plated on CSM agar lacking histidine and are incubated at 30 °C for 40 to 48 hours. Single colonies are selected and restreaked for single colonies on the same media. A culture from a single colony is grown in YPD and a genomic DNA is isolated for 5 evaluation by PCR reaction to verify the construction of the strain.

PCR primers for genomic verification are designed so that one primer (Primer 5') at one end of the gene is 5 prime to Primer 1 (above) used to generate the shuffled gene on the plasmid and on the same strand as Primer 1. Another primer (Primer 3') is designed which is 3 prime to Primer 4 (above) and on the same strand as Primer 4. PCR conditions 10 are as those described for Primary PCR (above). Another primer, specific for the plasmid sequence, 5'-ACCCCTGGCGCCCAATACG-3' (SEQ ID NO:23), is used in conjunction with Primer 3' to amplify DNA from the mutant strains, i.e., those that contain the shuffled genes. The product of this PCR reaction is typically 600 to 700 base pairs in length.

Wild type genomic DNA and Primer 5' and Primer 3' are used to amplify a 15 1 to 1.5 kb PCR product. This primer pair should not yield a PCR product from the mutant genomic DNA using these PCR conditions because the product would be too big to amplify (>7 kb).

#### Example 5: Assay for Reversion Frequency in Yeast Strains Engineered with Copper

##### **20 Repressible Genes**

The following assay was performed to assess the frequency with which a culture maintained under non-selective conditions will revert to a phenotype of non-sensitivity to exogenously added metal ions. Cultures of strains produced by two different methods in different strain backgrounds were grown in the absence of selection, then assayed 25 to determine what percentage of the cells in the culture were no longer sensitive to the addition of copper ions.

Two independent isolates of ZMY71 (ZM71 #1 and ZM71 #2) were used in this assay. ZM71 is derived from ZMY60, and its construction is described in Moqtaderi, Z. et al., *Nature* 383:188-191 (1996). The SUA7 gene was operably linked to an ANB1 promoter by 30 a single cross-over strategy in a strain in which ROX1 and UBR1 are activated by the addition of copper to the culture medium. The recombinant strains are maintained by

selection on media lacking uracil (-URA). It is known that in the absence of selection, spontaneous recombination results in a strain in which the URA3 gene is lost (reverting to a ura3 phenotype) and regulation of the SUA7 gene by the ANB1 promoter is lost, while wild-type regulation of SUA7 is restored.

5 Two independent yeast strain isolates (19SG1 and 19SG2) were also used. In these yeast strains the SUA7 gene in strain CUY106 was operably linked to an ANB1 promoter by the double cross-over strategy as detailed in Example 4. The recombination results in a strain that can be selected for on media lacking histidine (-HIS). Because of the method used to engineer this strain results in an insertion which does not contain any tandem 10 repeats of sequence, it should be less likely that in the absence of selection on -HIS media the strain would revert to a his3 phenotype or regain wild-type regulation of the SUA7 gene.

All strains were streaked from glycerol stocks to the appropriate selective media (uracil free media for ZM71 and histidine free media for the 19SG strains) and were grown for 72 hours at 30°C. Single colonies were picked and inoculated into 2 ml of 15 selective media and were cultured overnight in a rollerdrum at 30°C. The yeast cultures were microcentrifuged for approximately 5 seconds and the pellets were resuspended in two ml of YPD media (non-selective). The cultures were then grown 24 hours at 30°C in a rollerdrum.

Dilutions of each culture were plated to YPD and CSM plus 1 mM cupric 20 sulfate plates. Plates were incubated for 72 hours at 30°C and the colonies were counted.

YPD plate colony numbers reflect the total cells in the culture, while colonies on the CSM plus 1 mM cupric sulfate plates indicate revertants, i.e., cells which have become insensitive to the copper ion stimulus. Revertants are expressed in the table below as a percentage of total cells observed.

25

<u>STRAIN</u>	<u>REVERTANTS</u>
ZM71 #1	0.012%
ZM71 #2	0.22%
19SG1	0.00024%
30 19SG2	0.00042%

Not all reverions are due to genetic changes at the SUA7 locus. It is also possible that the copper stimulation of UBR1 or ROX1 gene expression can be ablated. However, since the control of these genes is identical in all the strains, any ablation of copper stimulation of these genes appear as background which all strains will share. The change in 5 the reversion frequency at 24 hours in the 19SG strains engineered according the methods of Example 4 demonstrates the improvements that can be achieved by altering gene expression in yeast according the methods disclosed herein.

**Example 6: Construction of Yeast Strains Containing CYC8-LexA Repressor Under 10 Copper Control**

The following procedures are performed to produce a yeast strain that expresses a heterologous repressor under copper control. Such strains avoid the potential problems of ROX1-based repressor strains, which include the pleiotropic effects and toxicities of ROX1 and metal ions. In a LexA-based repressor system, the addition of metal 15 ions represses only recombinant genes whose promoters have been engineered to contain the bacterial-derived recognition sequence for LexA (LexA operator). A CYC8-LexA fusion has been shown to repress the transcription of a yeast gene when the LexA operator sequence is placed adjacent to the promoter of the yeast gene (Keleher et al., *Cell* **68**:709, 1992).

This system comprises two components: (i) a yeast strain which, in the 20 presence of copper, expresses a CYC8-LexA fusion protein or a fusion protein between LexA and a fragment of ROX1 that lacks DNA-binding activity and (ii) a DNA fragment which renders any desired target gene repressible by LexA when introduced upstream of the start of the open reading frame. Notably, this can be achieved even if only a limited amount of sequence information is available.

25 A repressor fusion protein is constructed so that the DNA-binding domain of LexA (amino acids 1-87) is fused to the N-terminus of the entire CYC8 protein (amino acids 1-966) as well as 23 amino acids derived from the 5' untranslated region of CYC8. This hybrid protein is expressed from a conditionally "inert" locus, such as TRP1. Alternatively, a fusion protein is constructed so that the DNA-binding domain of LexA is fused to a ROX1 30 protein which has been mutated so that it no longer binds to ROX1 recognition sequences, such as those present in the yeast ANB1 promoter.

In order to render a particular gene repressible by the hybrid repressor proteins described above, a generic repressible promoter cassette is designed that can be inserted upstream of any gene. The promoter cassette consists of one to several copies of the LexA operator placed upstream of a UAS-containing yeast promoter, such as, e.g., CYC1, adjacent 5 to a gene such as HIS3 which can be used for positive selection. The insertion cassette can be produced by a single- or double-round PCR strategy by analogy to the method shown in Figure 2. In this case, the point of insertion is upstream of the promoter region and not at the translational start site (Figure 3).

The repressible promoter is modified so that it will integrate upstream of a 10 given yeast target gene as follows. Four PCR primers are designed based on limited sequence data flanking the 5' end of the gene of interest as shown in Figure 2.

- (i) Primer A is located 100-200 base pairs upstream of the beginning of the open reading frame of the gene, oriented toward the gene.
- (ii) Primer B is located 100-200 within the 5' end of the open reading 15 frame, oriented towards the 5' start of the gene.
- (iii) Primers C and D contain both sequences specific to each gene as well as sequences homologous to the generic repressible promoter cassette. Primer C contains at its 5'-most end sequences corresponding to the 3' end of the promoter cassette, in this case the 3' end of the CYC1 promoter. The 3' half of primer C contains sequences corresponding to 20 the 5' end of the open reading frame of interest. The 3' end of Primer D consists of the sequence complementary to the sequence just upstream of the gene of interest. The 5' half of Primer D consists of sequences complementary to the left-most end of the promoter cassette, in this case the 3' end of the HIS3 gene.

Typical sequences for primers C and D are:

25 Primer C: 5'-ACAAATACACACACTAAATAATGNNNNNNN-  
3' (SEQ ID NO:19)

Primer D: 5'-end of HIS3- NNNNNNNNNNNNNNNNNNN-3'

Two sets of PCR reactions are performed. In the first set, a fragment of DNA containing the gene of interest and 5' flanking region is used as a template and amplification 30 is performed using Primers A and D or Primers B and C. The resulting fragments are then included in a second round of PCR containing the promoter cassette, both initial PCR

products, and Primers A and B. This results in a larger fragment containing the promoter cassette flanked by pieces of DNA that will target the DNA just upstream of the gene of interest (Figure 3).

Following transformation into yeast and selection for integration, stable 5 integrants produced by sequence-specific recombination into the site of interest can be positively identified by PCR or Southern blot analyses. This strategy produces a conditional locus that is repressible by the LexA fusion proteins described above.

**Example 7: Demonstration of Cidal and Static Effects of Copper Ion-induced**

**10 Repression of Gene Expression**

Three different yeast cell strains were constructed based on the CUY106 strain. The yeast CDC15, SUA7, and ERG11 genes were rendered repressible by copper-ion addition to the growth medium as described above.

The viability of each strain was evaluated at several time points over an 8 hour 15 period after the addition of 1 mM copper sulfate to the growth medium by diluting the cells and plating them on YPD medium, without copper sulfate. Yeast colonies were counted after 48 hours of incubation at 30 °C in order to determine the colony forming units (CFU) per ml of original copper sulfate-containing culture medium at the time the yeast cells were harvested and diluted. The CFU/ml value was divided by the measured absorbance of the 20 original copper sulfate-containing growth medium at 600 nm ( $A_{600}$ ) at the time the aliquots were taken for dilution and plating onto YPD medium, yielding a viability index of CFU/ $A_{600}$ . The limit of detection for CFU/ $A_{600}$  is approximately  $1 \times 10^5$ . Figure 11 shows the results of the assay, demonstrating that repression of the expression of some genes, such as SUA7 and ERG11, is cidal, i.e., kills, the yeast cells, while repression of expression of 25 other genes, such as the CDC15 gene, only has a static effect on the yeast cells, arresting their growth but not killing them.

Claims:

1. A yeast cell comprising:
  - (i) a first gene encoding a transcriptional repressor protein whose expression is under the control of a metal ion-responsive element, wherein expression of said first gene encoding said repressor protein is stimulated by the addition of a metal ion to growth medium of said yeast cell;
  - (ii) a second gene encoding a subject protein, wherein expression of said second gene encoding said subject protein is controlled by a transcriptional control sequence whose activity is inhibited by said repressor protein; and
  - (iii) a third gene encoding a biomineralization protein, wherein said third gene is inactivated and wherein inactivation of said third gene enhances transcriptional response of said metal ion-responsive element to metal ions in said growth medium of said yeast cell.
- 15 2. A yeast cell as defined in claim 1, wherein said transcriptional repressor protein is the protein encoded by the ROX1 gene.
3. A yeast cell as defined in claim 1, wherein said transcriptional control sequence is ANB1 promoter.
- 20 4. A yeast cell as defined in claim 1, wherein said biomineralization gene is SLF1.
5. A yeast cell as defined in claim 1, further comprising a fourth gene encoding a protein that targets ubiquitin-containing polypeptides for degradation.
- 25 6. A yeast cell as defined in claim 5, wherein said fourth gene is placed under the control of a metal ion-responsive element.
- 30 7. A yeast cell as defined in claim 5, wherein said fourth gene is UBR1.

8. A yeast cell as defined in claim 5, wherein said second gene further comprises additional DNA sequences encoding peptides that target proteins for ubiquitin-mediated degradation, wherein said additional DNA sequences are fused in-frame to the gene encoding said subject protein.

5

9. A yeast cell as defined in claim 1, wherein said transcriptional repressor protein is CYC8-LexA.

10. A yeast cell as defined in claim 1, wherein said gene encoding said transcriptional repressor protein is ROX1-LexA.

11. A yeast cell comprising:

- (i) a first gene encoding a subject protein, the expression of said gene being under the control of a metal ion-responsive element, wherein expression of said first gene is stimulated by the addition of metal ions to growth medium of said yeast cell; and
- (ii) a second gene encoding a biomineralization protein, wherein said second gene is inactivated and wherein inactivation of said second gene enhances the transcriptional response of said metal ion-responsive element to metal ions added to said growth medium of said yeast cell.

20

12. A yeast cell as defined in claim 11, wherein said biomineralization gene is SLF1.

13. A method for repressing expression of a subject gene in a yeast cell to a predetermined level, said method comprising culturing a yeast cell as defined in claim 1 in the presence of metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression of expression of said subject gene.

30 14. A method for repressing expression of a subject gene in a yeast cell to a predetermined level, said method comprising culturing a yeast cell as defined in claim

7 in the presence of metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of repression of expression of said subject gene.

5           15. A method for activating expression of a subject gene in a yeast cell to a predetermined level, said method comprising culturing a yeast cell as defined in claim 11 in the presence of metal ions, wherein said metal ions are present in sufficient concentration to activate said metal ion-responsive element to a level which will result in said predetermined level of activation of expression of said subject gene.

10

16. A method for the introduction of a subject gene under the control a predetermined transcriptional control sequence into a yeast cell genome, comprising the steps of:

15 providing a shuffled gene fragment, wherein said fragment comprises a restriction enzyme cleavage sequence;

ligating said shuffled gene fragment into a vector, wherein said ligation results in said shuffled gene fragment being operably linked to a predetermined transcriptional control sequence;

20 cutting said vector with a restriction enzyme specific for said restriction enzyme cleavage sequence to yield a linearized vector; and  
transforming a yeast cell with said linearized vector.

17. The method of claim 16, wherein said transcriptional control sequence is the ANB1 promoter.

25

18. The method of claim 16, wherein said restriction enzyme cleavage sequence is a NotI sequence.

30           19. A method for the introduction of a subject gene under the control a predetermined transcriptional control sequence into a yeast cell genome, comprising the steps of:

(a) producing a first polymerase chain reaction product which comprises a top nucleic acid strand and a bottom nucleic acid strand and which comprises, in the 5' to 3' direction of the top strand, the ATG start codon of said subject gene, about 400 nucleotides which occur downstream of said ATG start codon in said subject gene, a restriction enzyme 5 cleavage sequence, and a nucleotide sequence which is identical to a sequence about 400 nucleotides upstream of said ATG start codon;

(b) producing a second polymerase chain reaction product which comprises a top nucleic acid strand and a bottom nucleic acid strand and which comprises, in the 5' to 3' direction of the top strand, a nucleotide sequence which is identical to a 10 sequence about 400 nucleotides downstream of said ATG start codon, a restriction enzyme cleavage sequence which is identical to said NotI restriction enzyme cleavage sequence in step (a), and about 400 nucleotides of 5' noncoding sequence of said gene immediately upstream of said ATG start codon;

(c) melting said first and said second polymerase chain reaction products 15 by heating to yield single stranded nucleic acid fragments;

(d) annealing said single stranded nucleic acid fragments;

(e) amplifying the nucleotide sequence encoded by said annealed nucleic acid fragments by performing polymerase chain reaction to yield a shuffled gene fragment;

(f) ligating said shuffled gene fragment into a vector, wherein said 20 ligation results in said shuffled gene being operably linked to a predetermined transcriptional control DNA sequence;

(g) cutting said vector with a restriction enzyme specific for said restriction enzyme cleavage sequence to yield a linearized vector; and

(h) transforming yeast with said linearized vector.

25

20. The method of claim 19, wherein said transcriptional control sequence is the ANB1 promoter.

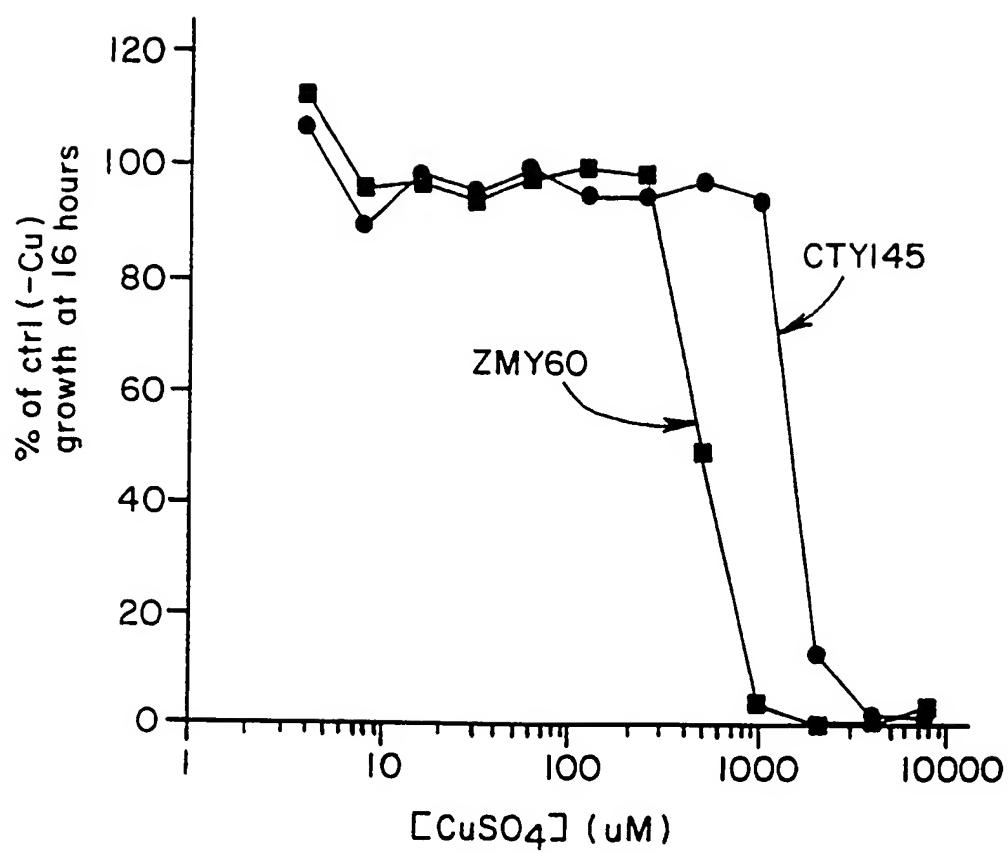
21. The method of claim 19, wherein said restriction enzyme cleavage 30 sequence is a NotI sequence.

33

22. The method of claim 13, wherein said metal ions are silver ions.
23. The method of claim 14, wherein said metal ions are silver ions.
- 5 24. The method of claim 15, wherein said metal ions are silver ions.

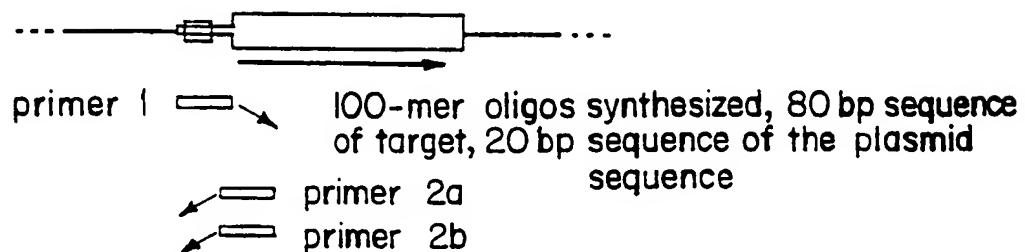
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FIG. 1

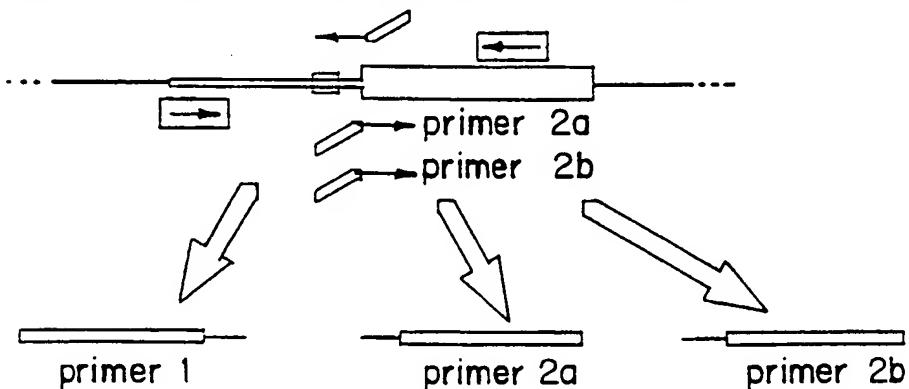


# FIG. 2A

## A. SINGLE ROUND PCR STRATEGY (OLIGOS SYNTHESIZED):



## B. DOUBLE ROUND PCR STRATEGY (OLIGOS PRODUCED BY PCR)



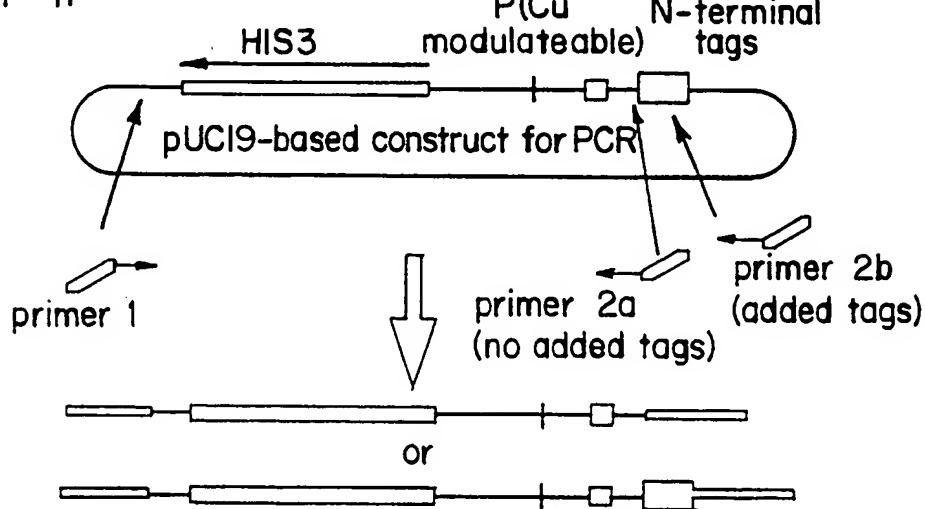
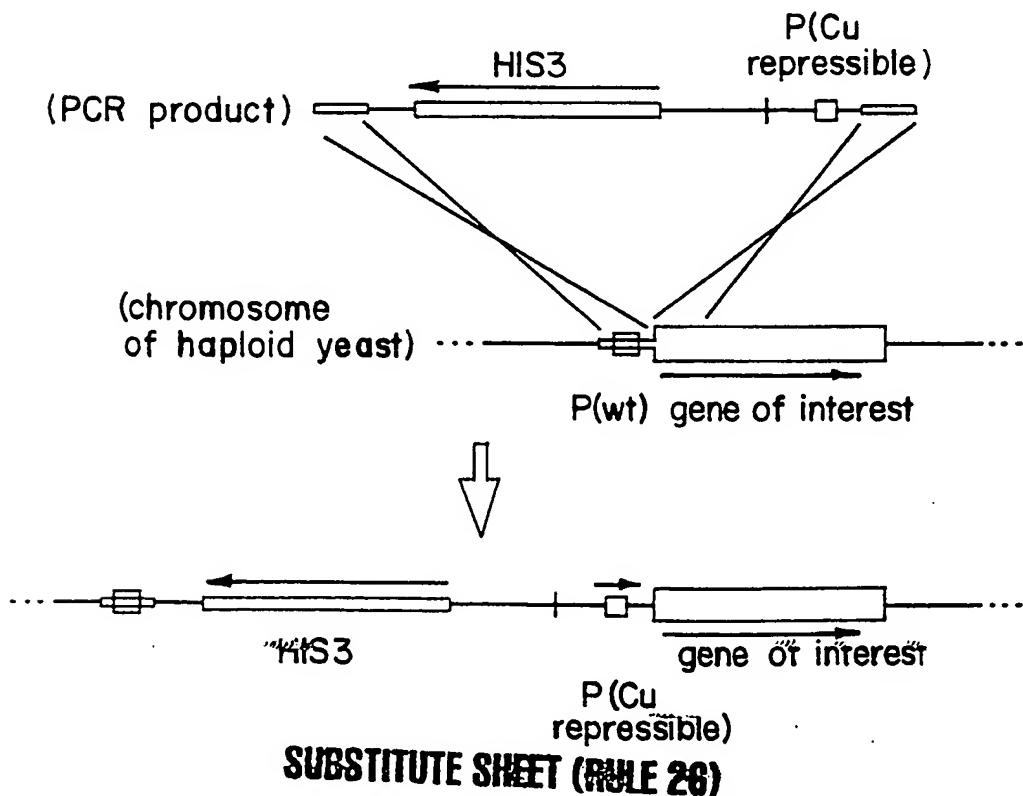
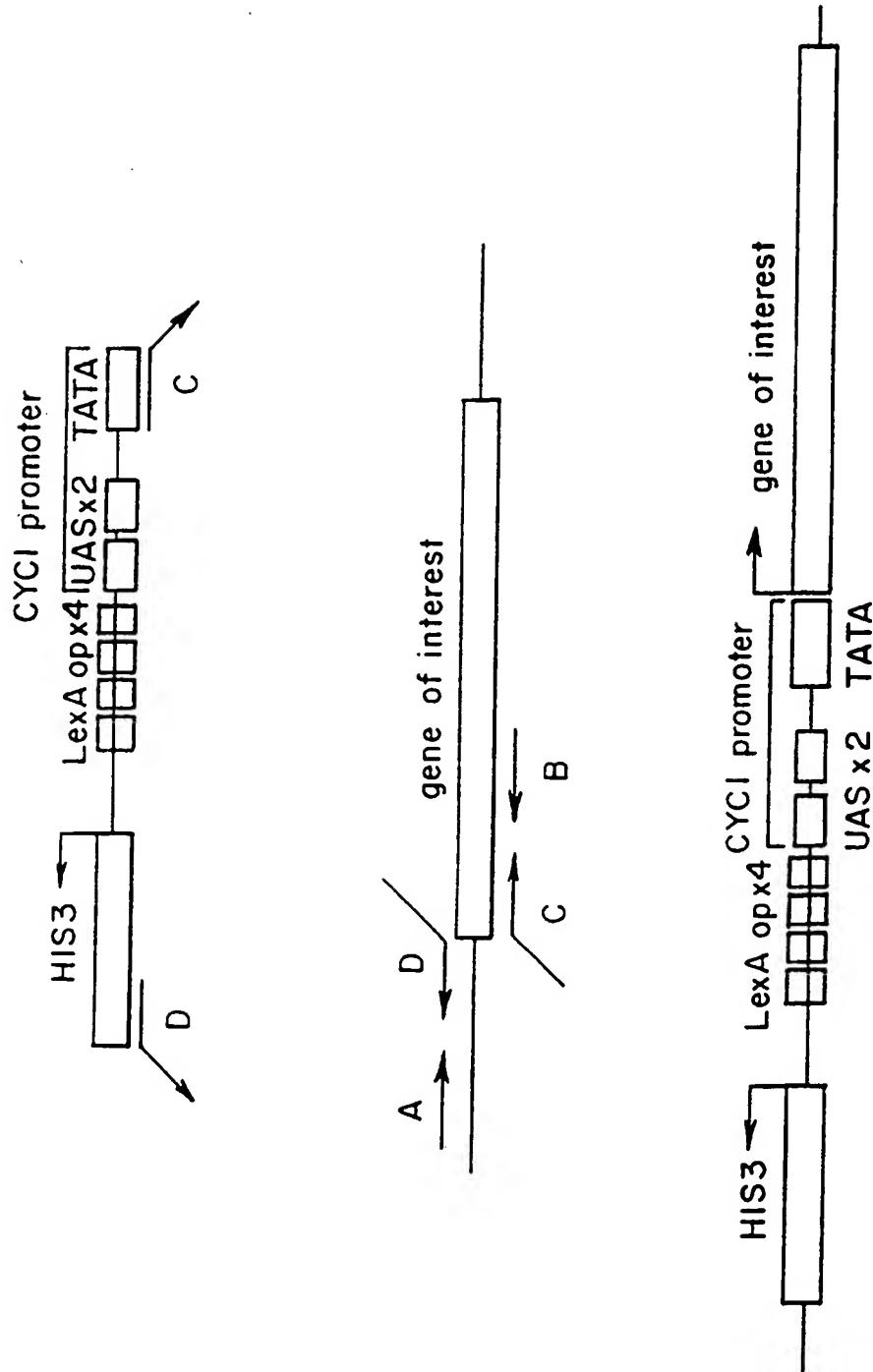
**FIG. 2B****II: TRANSFORMING DNA PRODUCED BY PCR WITH OLIGOS FROM STEP I:****FIG. 2C****III: TRANSFORMATION, RECOMBINATION**

FIG. 3



**FIG. 4A**

GAATTAAATCGAGCTCGGTACCGGTGATCTCGCTCGGCCACAAATCCCCCTGGATATCATTGGCC  
 TGTGAGGTATCGGCCGGTGGAACTACGGGAATTACTATGCAAACAAATTGGAAATCTGGTAG  
 GAAAACCTTGTTCAGAACCTGGCGATTGCTGACAAAGAAGAAAAGGGCCTATGTTGCTGCCCTC  
 TTTGTTGTTCTCGTATTGTCCTCGTATTGCTTGGTCTTCACTTGGCTAATGTAACGGTCTTAA  
 TATATAGTGCTCTTGCTTATTATTTCTCGTCTTCACTTGGCTAATGTAACGGTCTTAA  
 CAAAGTTTTTTTGCTCTTGATTTCCAGTTTAATGTTCTCAACTTCCCTCTCACCCCTCATTA  
 TTCAGAAATACTAATACCTAACACAAAGAACCGCAGTTAGACAAATCAACAAATGACTAGTA  
 GTTTCTGAAACCAAGGAAAGGTCAACAGAGGCAATAGACTCTTCATTGATCTTGC  
 TTGGCTTCCTGCAGTGGACGAGAACCTGGCCTTTGCTTAACCTCTCCTCAATTGGTTGTT  
 TCTCTTGATTTGAGCATCCAATTGCTTAATAGAGTCGTTGAATGTTGCTTCAACT  
 CAGCTTGGATCTTGATGATCCTCCCTGGTTTATCCTGTAACTCTTACGTTGCTTCAATT  
 TCGTTGACCTGGTGTGATCGATTGCTTCTTAATTAAACCGATTTCAGTGTCGATTTC  
 TTGAACGTTAACAGTGTCAATTCTGTTCTCAACGGAGACATCTGGGGCTTGAACCTGGT  
 GTTGGGAGGACATGGCAATTGGCTGGTTAGAAAATATGCTTATTACGTTGATAAAAGGAGGA  
 AGGTGAAATCAGTTCAAAATATGTGAATGAAACTGAACGAAAGAAATGACCAATGAGT  
 GGAGATGGAGGGCAAAATGAAAATGAAAAGGATGAACCTAAAGAAATAGAAATAGACT  
 GTACTTTAATGCTATGTTAACGCAACCAAGCAATTTCGAAACTCAATTGGCTTATAAATGTT  
 CGAGATAAAATGCGAATTACGTTCAACGTCGAGATCAGTTATTTTCAGTCACTCC  
 GCGGGTAAGGCAATTTCGGTACCCACCATCACATGTATAATGTTATTAGGCTTATTATG  
 TATGTTGTTGCTACTTTATGACGGTTATTACAAAGTTAGAATATTATCTTAAACAAATGCA  
 AGCCACGGCTTACGTTAGTGAAGTCAACAAATGGGGTCTGGGGCCGATTGCCTTCTCAATGCCAC  
 CAAAGGGATTTCGACGAAGTCACTCCTCATCTCAAAATTGGCTTACATTCAGGCTTTC  
 TTCCCCACCACTAGAACACAGGCAAGCTCGTTACATAATCCGTTCAAAATCGTGCATGCT  
 TTTCACAAACAGTTGTTCTGACGTTGGCATTAAGCTAACGTTGTAATAAACGTTCCAGCCAC  
 CCATTCTGTGATTAGTAAAACCTAACGGTTTACACGGCTTACGGGACACCAGGGCTCA  
 AGGGAGGCACTGGTCTTGGACACCAGGGCTCAAGGGAGGCACTGGTCTTCTCGACATCAC

A

## FIG. 4B

A

ATGAAAGTCAACCCCCAGGAAGTAAGGCACTTAATAATGGCACCCGCTTCTTAATGGCACCCGCTTCTGTTTTCGTTACCTAAGGGCAGTCAGTTA  
 TTTCATCCAGATAAACCCGAGGAAACTTCTTTCGTTATTGAGGCCAGCTCGTGAATGCTTAATGCTGAAACTGGTGTCCAT  
 TGAGGTATATTTCGTTATTGAGGCCAGCTCGTGAATGCTTAATGCTGAAACTGGTGTCCAT  
 GTGCCCTAGGTACGCAATCTCCACAGGCTGCAAAAGGTTCAGGCAATGTTACAGGCAATTGGTGTCCAT  
 CCCGCTAATTGGTCAAAAGTTTAATCTGTGCTTGCACCGCTCTGCTGTAACCTCAGTCAGTCA  
 TCGACTATCTGAGAAATTACTAGGAATAAGTGCCATGGTACAGGAAACCCGAGAAATGGCAATTCT  
 ACTCGGGTTCAAGCAACGGCTGCATAAACGGCTGTGGTGGCTAGACATATTGAAAGATAAGGATTAT  
 CATTCAAGTTTCAGGCAATAGTCTTATTCTGAAACTTGGATTATGGCTCTTTGGCTTAAAT  
 TTCGCCCTGATTCTTGATCTCCTTTAGCTTGATCTGACGTGGCCTTTCGACGCTGGCCTTGGCTTAAAT  
 ATTCTAGTCTTGGCTTGGAAACGGTTGAGCGGAAAAGACGCATCGAAATTGAGCTCGGAA  
 TTGGCATTATCACAATAATGAATAATACATTATAAGTAATATAAGCTTGTGATTCTTCGAAGAAATAACT  
 AAAAAATGAGCAGGCAAGAGATAAACGAAGGCAAAAGGACGGTATCGATATAATGAATCCTAAATCC  
 TCTACACCTAACGATTCCAAGACCCAAAGAACGCAATTCTGTTCAAGACAGCCTACACAGGAT  
 CTTAATAGACGAATGGGACCCGCTCAAGGTGTGGAAATAACCCATAATTCAAACATTCTGTTAAAGAA  
 TTGGTACGAAGTGGAAAGGGCTTACAACCCGGAAAGATAAGGCAACTGGCACACTGGGAAAG  
 GAGAAACTAGAACATGAAAGTAAGTATCCTGAATAACAAACAAATAACAGCCGGTAAGGAAGTCTAAAGAA  
 GAAGGCAACTACTTTGAAGGAAATCGAGCAACAGCAGGCAACAAACAGAAACAGAACAGCAGCAGC  
 AGAAACAGTCACAACCGCAATTACAACAGCCCTTAACAAACAAATAAGCTTCTGTTAAAGGAGGCA  
 CATTCTCTTCAACCATTCCCTCGGGTCAAGGCTCGAACAGCTATCAGTCCAAATTGAAACAATGA  
 TCTTAAGGAGGTGCCTATTCTGTTAAACTATCTAACTATGTTCTCCAGATCCTCTAG  
 TCGACCTGCAGGCATGCAAGCTGGCTTAATCATGGCTATAGCTGTTGAAATTGTTA  
 TCGCTCACAAATTCCACACAAACATCGAGCCGAAAGCTAAAGTGTAAAGCCTGGGTGCCTAAT  
 GAGTGAGCTAACATCACATTAAATTGCGTGTGGGATCCACATTAGTCTAGGCTGAAAGCTGGCTAATCGGTCA  
 TGCCAGGGGGATCCACATTAGTCTAGGCTGCAAGCTGGCTAATCGGTCA  
 GCTGTTCCCTGTGTGAATAATTGTTATCCGCTCACAAATTCCACACAACTACGAGCCGGTCACTGCC  
 AGTGTAAAGCCTGGGTGCCTAATGAGTGAGCTAACATTAATTGCGTGTGGCTAATCGGTCA

B

## FIG. 4C

B

GCTTTCCAGTCGGAAACCTGCTGCCCCAAGCTGCAATTAAATGAAATCGGCTTCCGGCTTCTCGGCTTCTCGCTCACTGACTCGCTGGCTCGGGCTCGGCTCGGCT  
 CGGGCGAGGGTATCAGCTCAAGGGGTAATAACGGTTATCCACAGAATCAGGGATAAC  
 GCAGGAAAGAACATGTGAGCAAAGGCCAGCAAAGGGCAGGAAACGGTAAAGGGGATTAAC  
 GCGGTTTTCCATAGGCTTCCATAGGCTTCCGGCTTACCGGATAACCTGTCGGCATCACAAATCGACGGCTCAAGTCAGAGGT  
 CCTGTTCCGACCCCTGGCGCTTACCGGATAACCTGTCGGCTTCCGGCTTCCGGCT  
 TTCTCATAGCTCACGGCTTAGGTATCTCAGTTAGGTCTGGCTGGCTGGCTGTG  
 TGCACGAACCCCCGGTCAAGAGACTTACGGCAACTGGCAAGCCACTGGTAACAGGATTAGCAGAGGAGGT  
 CGGTAAGAACAGACTTACGGCAACTGGCTGGGGCTTAACACTACGGCTACACTAGAACAGGT  
 TGTAGGGGGTGTACAGAGTTCTTGAAAGTGGTGGGGTCAAGGGCTTACACTAACAGGACAGTAT  
 TTGGTATCTGGGCTCTGGCTGAAGGCCAGTTACCCCTGGAAAGGGGTTCTGGGTAGCTCTGGATCCGGC  
 AAACAAACCCGGCTGGTAGCGGGTCTGGGTTCTACGGGGTCTGACGGCTCAAGTGGAACGAAACTCAC  
 AGGATCTCAAGAACAGATCCTTGGATCTTCTACGGGGTCTGGCTTACCCATAGTTGGCTGACTCCCCGTG  
 GTTAAGGGATTGGTCAATGAGATTACAAAGGATCTTCAACCTAGATCCTTAATTAAGGAA  
 TGAAGTTAAATCAATCTAACAGTATAATGAGTAAACTTGGCTTACCCATAGTTGGCTGACTCCCCGTG  
 CAGTGAGGGCACCTATCTCAGGGATCTGGCTTACCCAGTGGGGCTTACCATCTGGGGCAATGATA  
 TGTTAGATAACTACGGATACTGGGGGGCTTACCATCTGGGGGGCTTACCATCTGGGGCAAGCTAGAGTAAGTA  
 CCACGGCTCAGGGCTCCAGATTATCCGGCTTACCCAGTGGGGGGCTTACCATCTGGGGCAATGATA  
 TGGTCCCTGCAACTTATCCGGCTTACCCAGTGGGGGGCTTACCATCTGGGGGGCTTACCATCTGGGGCA  
 GTRTCGCCAGTTAATAGTTGGGCAACGGTGGCCATTGGCTACAGGGCATCGGCTCAGGGCTCG  
 TCGTTGGTATGGCTTCAATTCAAGGGCTCCGGTTCCGGATCGTCAATTCTTACTGTCATGCCATCCGGTAAGATGC  
 GTTGTGCAAAAGGGTTAGCTCCGGCTCGGTCTCCGATCAAGGGGACTGGCTTACATGATCCCCAT  
 GTTATCACTCATGGTTATGGCAAGGCATGGCTCAATTCTTACTGTCATGCCATCCGGTAAGATGC  
 TTTCTGTGACTGGTGAGTACTCAACCAAGTCAATTCTGAGAATAAGTGTATGGGGGACCCGAGTTG  
 CTCTGCCCCGGCTCAATAACGGGATAATAACGGGCCACATAGCAGAAACTTTAAAGTGTCTCATCA  
 TTGGAAACGGTTCTCGGGGAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATG  
 TAACCCACTCGTGGCACCCAACTGATCTTCAGGCATCTTACTTACCCAGTCTGGGTGAGC

C

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## FIG. 4D

C

AAAAACAGGGAGGCAAAATGCCGCAAAATAAGGGAAATAAGGGACACGGGAAATGTGAAATACTCA  
TACTCTTCCTTTCTAATATTATTGAGGCATTATCAGGGTTATTGTCATGAGGGATAACATA  
TTGAAATGTATTAGAAAATAACAAATAAGGGTTCCGGCACATTCCCGAAAGTGCCACC  
TGACGGTCTAAGAAACATTATCATGACATTAAACCTATAAAATAGGGTATCACGGGCCAG  
CTTTCAATTCAATTCAATTCCATTTTATTCTTGTATTTCGTTCTTGAATTT  
TTTGATTCGGTAATCTCGAACAGAAGGAAGAACGAAAGGACAGACTTAGATTGGTATA  
TATACGGCATATGTAGTGTGAAGAAACATGAAATTGCCCAGTATTCTTAACCCAAACTGCACAGAA  
CTTTGATTAGGAAACGGAAAGATAAATCATGTCGAAGCTACATATAAGGAACGTTGCTACT  
CAAAACATGAGGAAACGGAAAGCTATTAAATCATGCCAACGAAAGCAAACAAACTTGTGTGC  
CATCCCTAGTCTGTGCTGCCAACGCTATTAAATCATGCCAACGAAAGCAAACAAACTTGTGTGC  
TTCATGGATGTTCGTACCAAGGAATTACTGGAGTTAGTTGAAGGCATTAGGTCCCCAAATT  
GTTTACTAAACACATGTGGATATTCTGACTGATTTCATGGAGGGCACAGTTAACGCCGCTA  
AAGGCATTATCCGCCAAGTACAATTTTTACTCTCTCGAAGAACAGAAAATTGGTACATGGTAA  
TACAGTCAAATTGCACTCGCAGTACTCTGGGGTGTATACAGAAATAGCAGAATTACGAAT  
CACACGGTGTGGCCAGGTATTGTTAGGGTTGAAAGCAGGGCAGAAGAACGAAAGTAACAAAG  
GAACCTAGAGGCCCTTTGATGTTAGCAGAATTGTCATGCCAAGGGCTCCCTATCTACTGGAGAATA  
TACTAACGGTACTGTGACATTGCAAGGGACAAAGATTGTTGATATTGCTTATGGCTCAA  
GAGACATGGGTGAAAGAGATGAAGGGTACGATTGGTGTATGACACCCCCGGTGGTTTAGAT  
GACAAGGGAGACGGCATTGGGTAACACAGTATAGAACCGTGGATGATGTGGTCTACAGGATCTGA  
CATTATTGCTGCACTAAACTCACAAATTAGAGCTTCAATTAAATTATCAGTTATTAC  
ACAGAAAAGCAGGCTGGGAAGGCATATTGAGAAGGACTATTGCAAGGGAAAGGATGCTAAGGT  
TAAGTAAATGCATGTATACTAACGGTCACTAAACTCACAAATTAGAGCTTCAATTAAATTATCAGTT  
CGCCCTTCCGTTCCGGCGTTCCGGTGTGGGCTGGCTTAACATGGGGCATAGGGCAGATTGTA  
ACGGTACAGCTTGTCTGTAAGGGGATGCCGGGAGACAGGGCAGTGGGGCATGGGG  
TGTGCGGGGTGTGGGGCTGGCTTAACATGGGGCATAGGGCAGATTGTAAGGG  
ATATGCGGTTGAAATAACGGCACAGATGGTAAAGGAGAAAATACCGCATGGGG  
TTCAAGGGCTGGCAACTGTTGGAAGGGCGATGGGTGGGGCTCTTCGCTTATTACGCCAGCTGGC  
GAAAGGGGGATGTTGGTAAAGTGGGCAAGGGGATTAAAGTGGTAAACGCCAGGTT  
GTAAAACGACGGCCAGT

## FIG. 5A

GAATTCAATTGGAGCTCGGTACCCAGTGCCACACCAAAAGTCGAAAGAACCAAAGAAATAA  
 GGTACTAACCCAGGGCTACTAAGACCAACGGAGATTGCCACGAAACTAGAGGAACCAAATTGTAAG  
 CATAAGCTTAATTCCGGTTTCAGCGATTCAAACTAATAATAATAAGAAAAGATATACTCATATAAAACGTTATAA  
 AATTAATAACGGGTAAAGTGTAGAAAGCTTATTTCCTCCTGGCGATTGAATT  
 AACTTGGCAGATAAGTGCACCATAGGCCAACTACCCAGTGGCAAACAGTTTGATAAACGCCACATCAAC  
 GAGCGAGTAAAGACCTTGGTACATTAAAGGAAACATATAATTGTTTCAATTGCTAGACCCCTTTA  
 GTCTCACCTCAATAAAACTGCTTTATTCCCTCATGGCTTTATTCTTAATTGGCATCCTGCTTCTGCCCTCCTTCT  
 TGAAACTGGCATTAAACAAAGCAACTATTAAATAGTAGCATCCTGGCATCTTGGACAAAGGCAGT  
 ATTGGGATAACATTAAAGTTTACCCACCTTTCTTCTTCTGAACTTCCCTCACTGAAGTCCCTAATCTTACAGGTACACACA  
 TGAAGTTTACGTATCCCTATTAGTTGACTATTAGTTGACTTACCTGAAACAAAGAAAGGTACCCAGAGGCAATAGACTCT  
 ATTACATAGAACATCCAACACTAGTAGTTTCTTGTGAACTTCCCTGAGTGGGAGAAACTTGGCCTTAACCTCTCC  
 TCAATTCTCATGATTCTTGTGGCTTCTGCAGTGGGATTCAGTGTGGATTTCAGTGTGGATTTCAGTGTGGATTTCAGTGTGG  
 CAATTGGTTGTTCTCTTGATTTGAGCATCCAATTGCTTAATAGAGTCGTGAATGTGCTTCTACTTACGTTCTTGTGG  
 GGTTTCAAGTCAGCTGGATCTTGATGATCTCCTGTTGATGTCCTTACCTGTATACTCGTGAATTACGTTCTTGTGG  
 GTGGTATCGTTGACCTGGTGGATTGCTTCTTAATTAAACCGATTTCAGTGTGGATTTCAGTGTGGATTTCAGTGTGG  
 CAATTGGCTTAAGAGGTCCAATTCTGTCTAACGGAGACATCTGGGCTTGAACCTGGCTACTTTAATGCTATGTT  
 ATTGAACGTTAAGAGGTCCAATTCTGTCTAACGGCTTAAATGCTATTACGTTGATAAAAGGAGGAAGGTGAAT  
 GGAGGACATGGCAATTGGCTGTGTTAGAAAATATGCTATTACGTTGATAAAAGGAGGAAGGTGAAT  
 CAGTTCAAATAATGTAATGAAATGAAACTGAACGAAAGAAATGACCCAGAATGAGTGAATAATGGAGATGGAGGGGC  
 AAAATGAAAAAAAGGATGAAACCTAAATAAGAAAATAGACTCCGGCTGCTACTTTAATGCTATGTT  
 AACGCCAACCAAGCAATTTCGAACACTCAATTGGCTTAAATGCTTACGTTGCTACTTTAATGACGGGTATTTC  
 TCAACGTCGTCGAGATCAGTTTCAATTAAACATGGCAGTAGCCACGCTTACGTTAGTGAATTGGGTCT  
 CCATTACACATGTATAATGTTATTATGCTATTATGCTTAAATGCTTACGTTAGTGAATTGGGTCT  
 AAGTTAGAAATTATCTTCAATTAAACATGGCAGTAGCCACGCTTACGTTAGTGAATTGGGTCT  
 GGCCCGATTGCCCTTCTCAATTGGCAGTAGCCACGCTTACGTTAGTGAATTGGGTCT  
 GTTCTTAAGGCCCTGGCTTCTGACGTTGGCATTAGCTAACAGTGTATTTCAGTAACTGGCTTGAATAAA  
 CGTCCAGCCACCCATTTCGTGATTAGTAAAACACTTAACGTTTATCAACGTAATAATGGGCAGA  
 AGTTCGAGGGCCCCACTGCTGTCTGGACACCACAGCGTCAAAAGGAGGAGCAGTTCTCGACATCA  
 A

## FIG. 5B

A

CAATGAAGTCAACCCCAAGGAAGTAAGCGGCTTCTAATAATGGCACCCGATATTGTGAGGGTCAGTTATTTC  
 ATCCAGATATAACCCGAGGAAACTTCTTAGCGCTCTGTTTCGTAATGGCTAACATAAGGCAGTTCATGAGGTATA  
 CAATCTCCACAGGCTGCCAGCTCGTGAATGCTTAATGGCTGAAACTGGGTGTCCATGTCGCCCTAGGTACG  
 GTTTAATCTGTGCTTGTCCACAGCTCTGTCTGTAACCTTCAGTTCAATGGCAATTGGCTTAATTGGTCAACAA  
 GGAATAGTGCATGGTACAGCAACCCGAGAATGGCAATTGGCAATTGGCTTAATTGGTCAACCTTCAGACTATCTGAAAGAAATTACTA  
 TGTGTTGGCTAGACATATCGAAGATAAGGATTATCAAGTTTCAATTCAAGTTTCAAGGCAATGTCCTTATTCTG  
 GAACTTGGGATTTATGGCTCTTTGGTTAAATTGGATCTGAATTCTAGTCTTGTGATCTGCCATTGGATTACATTA  
 GCCTTTTCTTGTGATCTAGTCTTGTGAAATTGGATCTGAATTGGATCTGAATTGGATTGGCATTATCACATA  
 CGAATTTCGAGCTCGTTAGCGATTGGGATCTGAATTGGGATCTGAATTGGGATCTGAATTGGGATCTGAATTGGG  
 CTTCTGAAAGAATATACTAAAGGAGGCAAGATAAACGAAAGGCAAAAGGACGGTATCGATAAGCTTGG  
 GAATTCAAAATGCCCAAGGAAGGGCCATATGTACCCATACGACGTTCCAGACTACGCTTCT  
 TGGTGGTTCTAGCCCAGCTTGATATCGAAATTCCCTGCAGCCCCGGGATCTAACATGTCGGTTGCTGA  
 TGATGATTAGGATCTTACAAGGTCACATTAGGAGAACACTGAGGTCTATTCATAACCTCCCCTATT  
 AGGTATAAGGAGGGCTTACTGAAAGGGCTGACATGAGCAGGCCCTAAAGAGTTCAATTACAGATA  
 TATACTTTGTCAATTCTAACAGGGAGAAGCTTACCTACTTCAATTCAATGCTCATCCAAAACAAAATT  
 ATCTAACCCAGAGCTTACTGTTTCTGACAGTTAGAAGATGCTGTGGATATTGATAAGATAACATCT  
 CAACAAACTATTCCGTTTATAAGATAAGTCAAGGAAACCTTGATAGGTGTCATGAGTGTGGTGGCGATGATACTTG  
 ATTGTGGGAGGAATTCAAATAAGGGAAACCTTGATAGGTGTCATGCTGTTCCCTGTGAAATTGTTATATGTA  
 TGTGCTTGTATTCAAGCTTGGCGTAATCATGGTCATAGCTGTTCCCTGTGAAATTGTTATATGTA  
 GAATTGATAATCAAGGCTGGCGCTAACCTGGGGAAACCTGCTGGCCAGGGGGATCCACTAG  
 ATTCCACACACATACGAGCCGGAAAGCATAAAGTAAAGCTGGGTGCTTAATGAGTGAGCTAACCTCA  
 CATTAAATTGGCTTGGCGCTAACCTGGGGAAACCTGCTGGCCAGGGGGATCCACTAG  
 TTCTAGAGTGCACGGCATGGTCAAGCTGGGTAAATCATGGTCTGTTCCCTGTGAAATTGTTAT  
 CGGCTCACAAATTCCACACACATACGAGCCGGAAAGCATAACACACATACGAGCTGGGTGCTTAATGAGTG  
 GCTAACTCACATTAAATTGGCTTGGCGCTCACTGCCGGCTTCCAGTCGGAAACCTGCTGCCAGCTGCA

B

## FIG. 5C

B

TTAATGAAATGGCCAACGGCGGGAGAGGGCTTGGGTATTGGGGCTTCCGGCTCTCGCTCACT  
 GACTCGCTGGCTGGTCGGTTGGCTGGGAGGGCTGGTACAGCTCACTAAAGGGGTAATAACGGTTAT  
 CCACAGAAATCAGGGATAAACGCAGGAAGAACATGTGAGCCAAAGGCCAGGAAACCGTAA  
 AAAGGGCCGGCTGGCTGGGTTTTCCATAGGCTC'GGGGCTTGGCTGGGAGCTAACAAAGGGTAA  
 AGTCAGAGGGTGGGAAACCCGACAGGACTATAAGATAACCAGGGTTGGGAGCTAACAAAGGGTAA  
 GCTCTCCCTGGACCCCTGGGGCTTACCGGGATAACCTGTCCGGCTTGGCTGGGAGCTGGGCT  
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 C

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## FIG. 5D

C

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## FIG. 6

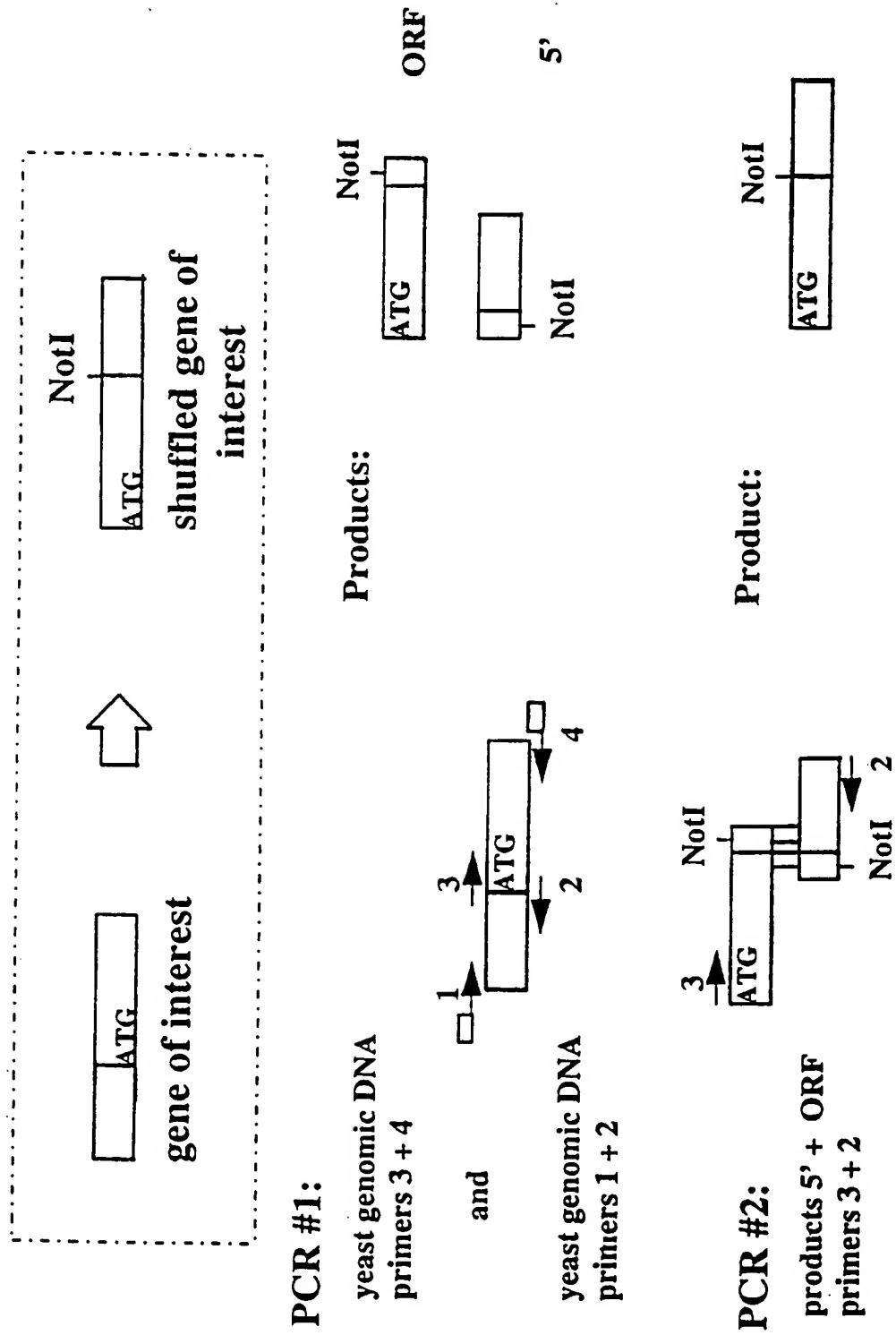
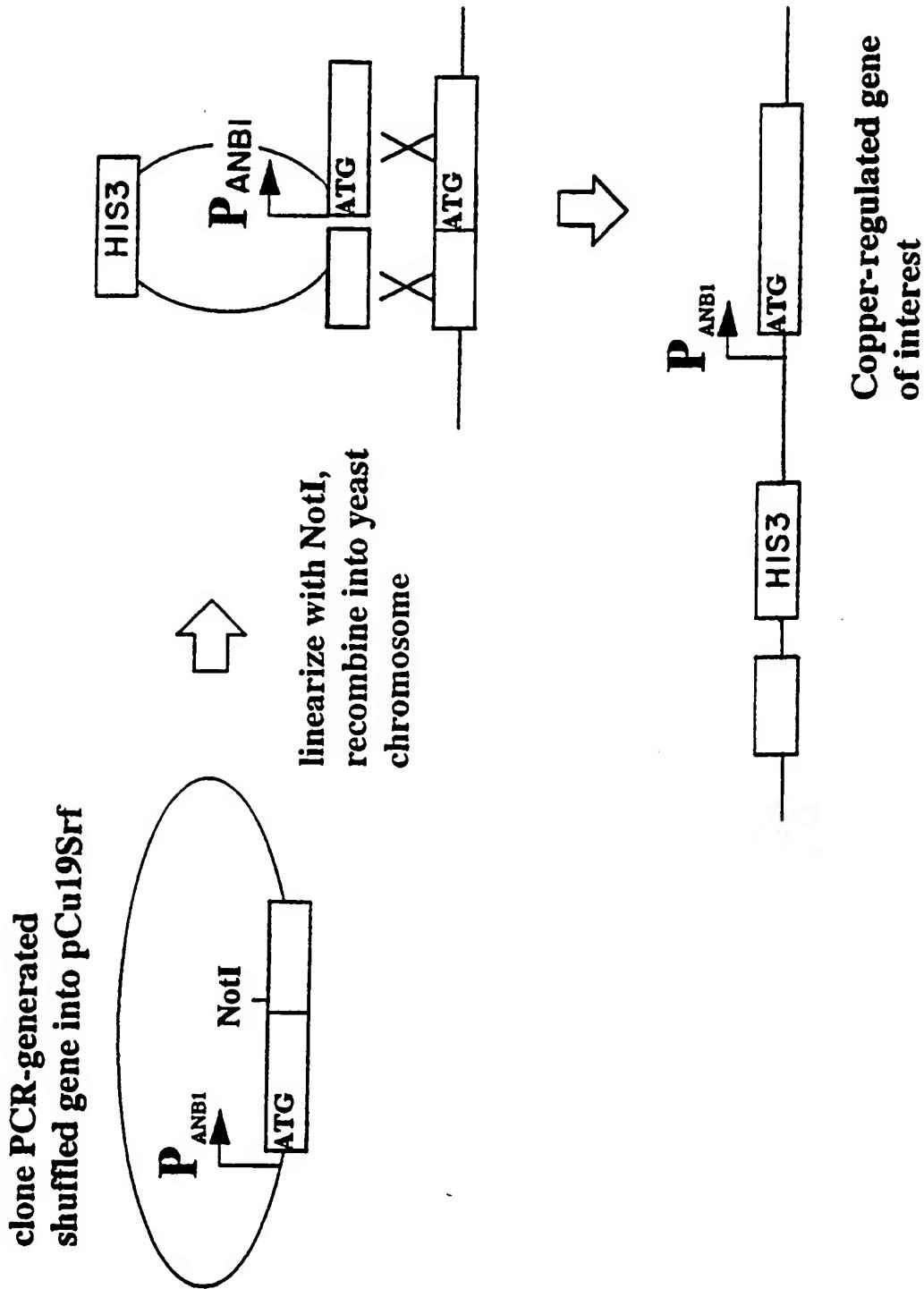


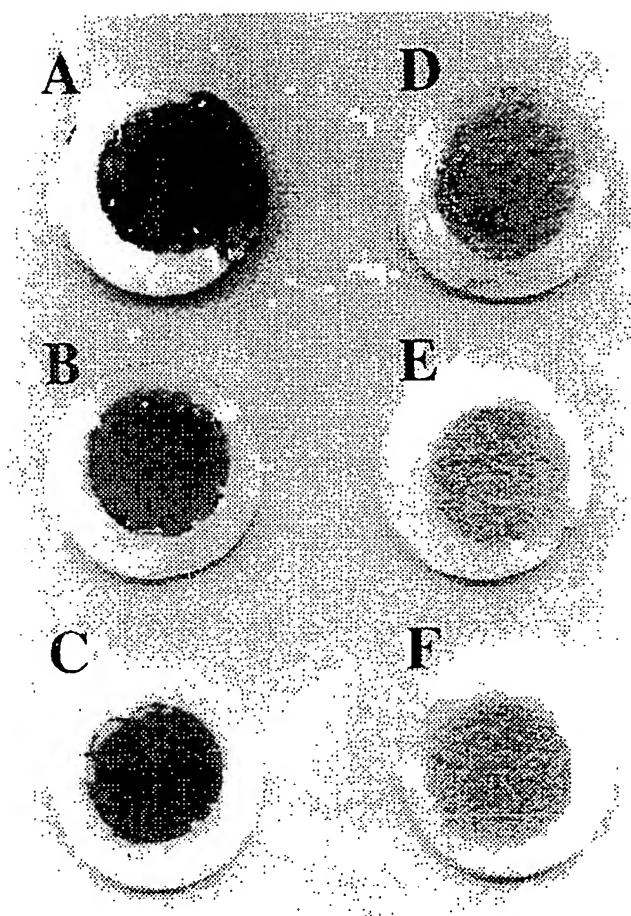
FIG. 7



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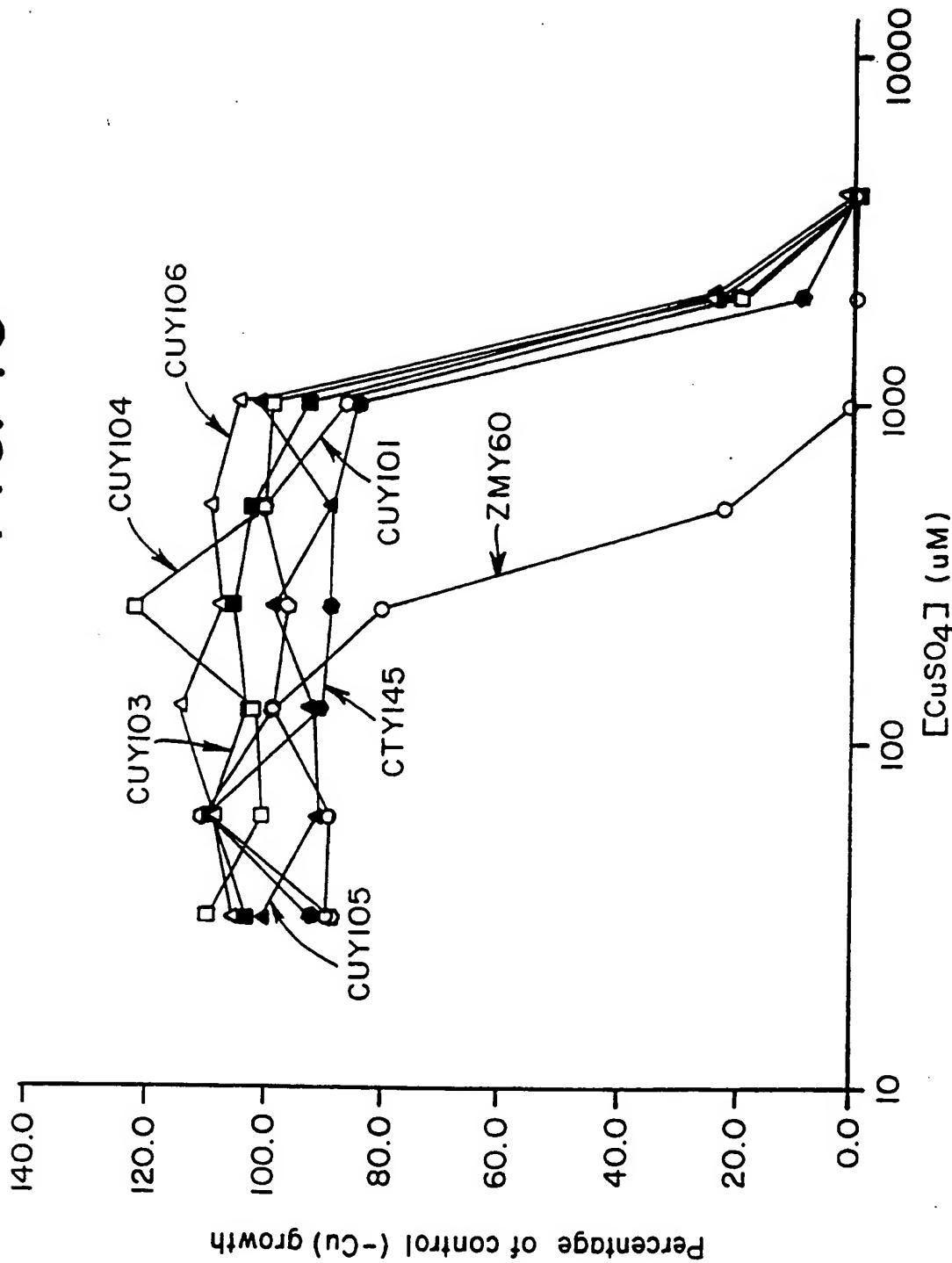
FIG. 8



**FIG. 9**

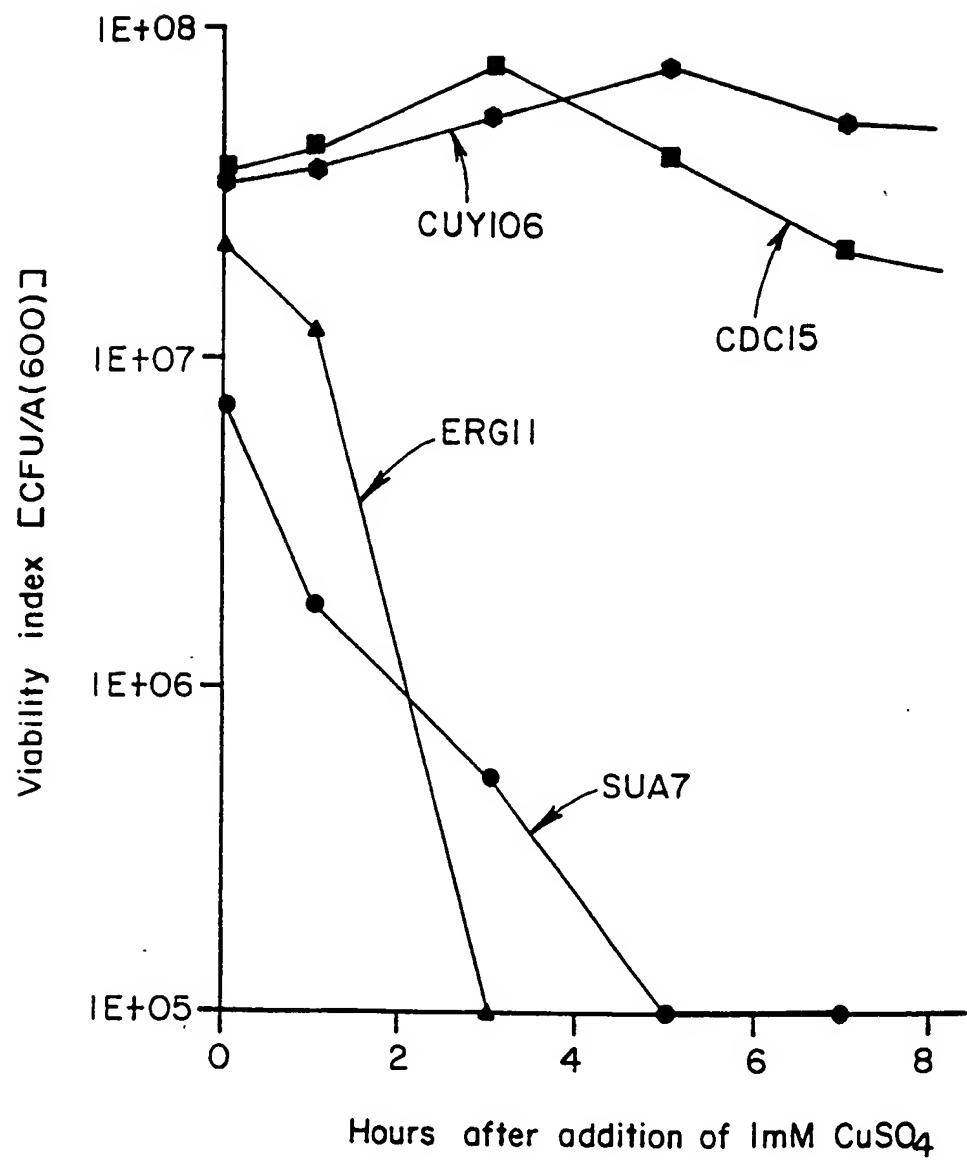
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FIG. 10



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FIG. 11





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18

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/17592

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) C12N 1/19, 15/63, 15/66, 15/67, 15/81  
US CL 435/69.1, 91.1, 172.3, 254.2

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 91.1, 172.3, 254.2

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS; DIALOG DATABASES; BIOSIS PREVIEWS, MEDLINE, WORLD PATENT INDEX, CA SEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	YU, W. et al. Identification of SLF1 as a New Copper Homeostasis Gene Involved in Copper Sulfide Mineralization in <i>Saccharomyces cerevisiae</i> . May 1996, Vol. 16, No. 5, pages 2464-2472, see entire article.	1-15, 22-24

Further documents are listed in the continuation of Box C.

See patent family annex.

- \* Special categories of cited documents:
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "E" earlier document published on or after the international filing date
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "&" document member of the same patent family

Date of the actual completion of the international search  
08 NOVEMBER 1998

Date of mailing of the international search report

15 DEC 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer  
JOHNNY F. RALEY II, PH.D. *for*  
Telephone No. (703) 308-0196

Facsimile No. (703) 305-3230